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Our studies, together with work carried out in other laboratories, have demonstrated that essentially all breast cancer cell lines and tumor tissues have defects of varying degrees in the cyclin D1-Rb-p16 regulatory pathway that controls cell cycle progression at the G1/S boundary. The most common defect in cell lines we observe is the expression or overexpression of cyclin D1 in the presence of Rb, accompanied by a failure to express p16. Expression of moderate amounts of p16 in breast cancer cell lines with this type of defect suppresses the transformed properties of the cells, preventing growth in soft agar and blocking formation of tumors in nude mice, indicating that the fundamental defect in these cell lines appears to be the failure to express p16. The most common defect in breast tumor tissues is the overexpression of cyclin D1 in the presence of both Rb and p16. The coexpression of Rb and p16 in these tumors suggests that abnormalities in cell cycle control may be minimal. However, in a subset of breast cancer cell lines also coexpressing Rb and p16, we have found that the fundamental defect in cell cycle regulation involves an insensitivity to the inhibitory actions of p16. These findings suggest the possibility that this unusual defect in cell cycle regulatory control may also be present in breast tumors which coexpress Rb and p16.

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INTRODUCTION

The initial advances made in the late 1980s and early 1990s in our understanding of the regulation of cell cycle progression provided a new perspective on the nature of the cellular defects which combine to produce malignant growth. These advances began with the identification of the cyclin-dependent kinases (CDKs) as the major drivers of cell cycle progression in all eukaryotes. The first indication that defects in cell cycle regulation contribute to malignant transformation of cells came with the discovery that the hepatoma virus integrates into the cyclin A gene in human hepatocellular carcinoma (Wang et al., 1990), and that the PRAD1 gene, which is overexpressed in parathyroid tumors, encodes cyclin D1 (Motokura et al., 1991). The association of cyclin D1 overexpression was of special interest, since D-type cyclins had been shown to play a role in regulating the G1/S transition in cells, by virtue of their ability to promote phosphorylation and inactivation of the retinoblastoma gene product, Rb, a tumor suppressor. During normal progression through the cell cycle, phosphorylation and inactivation of Rb by cyclin D1-CDK4/6 releases bound transcription factors necessary for transition of the G1/S boundary. Defects in Rb expression and regulation were known to be associated with a number of tumor types in addition to retinoblastoma, giving an early indication that loss of regulation at this point is important in the development of cancer.

The cyclin D1 gene was found to be present at a locus on chromosome 11, known as the BCL-1 region, which was translocated or amplified with varying degrees of frequency in a number of human cancers, including breast cancer (Schuuring et al., 1992). Further studies of cyclin D1 expression in breast tumors showed that cyclin D1 mRNA overexpression occurred in approximately half of a large number of breast tumors examined (Buckley et al., 1992). In our own laboratory, we had undertaken a study of cell cycle regulation in small cell and non-small cell lung cancer, and had found a consistent pattern of defects associated with each type. Small cell lung cancer cells invariably failed to express Rb, while non-small cell lung cancer cells overexpressed cyclin D1, indicating that the two types of lung cancer cells employed alternate mechanisms for overcoming cell cycle regulation at the G1/S boundary (Schauer et al., 1994), and suggesting that overcoming cyclin D1-Rb regulation by one mechanism or another might be a necessary step in oncogenesis.

Against this background we proposed to determine whether cyclin D1 protein itself is overexpressed in breast cancer cell lines and tumors, and to directly determine whether reduction of cyclin D1 expression using antisense technology reverses the transformed properties of breast cancer cells. We also proposed to undertake a comprehensive investigation of whether defects occur in breast cancer cells in the many other cell cycle regulatory components then known to exist. At the time of this proposal no measurements of cyclin D1 protein itself in breast cancer cells had been reported, and earlier studies of Rb expression in breast cancer had been carried out and interpreted in the complete absence of an understanding of the role of cyclin D1-Rb in cell cycle regulation. However, while this proposal was under consideration for funding, the potentially great importance of the cyclin D1-Rb regulatory system in malignancy became widely appreciated, and several laboratories quickly reported frequent overexpression of cyclin D1 protein in breast cancer

cell lines and tissues. However, none of these laboratories had carried out the comprehensive investigation and comparison of the multiple components known to contribute to cell cycle regulation that we had proposed. During the course of carrying out this investigation, knowledge of the components and mechanisms involved in overall cell cycle regulation has expanded enormously. In particular, based on studies in many laboratories, it is now clear that essentially all malignant cells contain one or more defects in the cyclin D1-Rb regulatory point at the G1/S boundary in the cell cycle, sometimes known as the restriction point (reviewed in Bartek et al., 1997). Defects in other aspects of cell cycle regulation, on the other hand, are not commonly associated with cancer. This knowledge represents a major advance in our understanding of the fundamental defects which contribute to the malignant state of cells.

We report here the results of our investigation, which has extended considerably beyond the specific aims and statement of work originally proposed. These extensions include an investigation of the mechanisms responsible for defects in the expression of key cell cycle regulatory proteins in cancer cells, and an extensive study of the contribution of defects in CDK inhibitor protein p16 (unknown at the time of the original proposal) expression and function to the malignant state of cells. The latter study became a major focus of the entire project and led to the discovery of an unusual defect in cell cycle regulation in a subset of breast cancer cell lines, which is not revealed simply by examining the level of expression of regulatory proteins. The presence of apparently minimal defects in cell cycle regulatory protein expression in the majority of breast cancer tissues we have examined suggests the possibility that this unusual type of defect may play a significant role in breast cancer incidence and oncogenesis.

RESULTS - SECTION I

ANALYSIS OF CELL CYCLE REGULATORY DEFECTS IN BREAST CANCER CELL LINES AND TUMOR TISSUES.

Expression of cell cycle regulatory proteins in breast cancer cell lines.

In order to investigate cell cycle regulatory defects associated with breast cancer, we first established a panel of 12 available breast cancer cell lines derived from various types of breast tumors, representing both primary tumors and metastatic deposits. These included carcinoma, adenocarcinoma, ductal carcinoma and medulla carcinoma. For comparison, we also established 2 available non-tumor immortalized breast epithelial cell lines (Table 1).

Cells grown in culture were harvested for protein extraction at 50-70% confluence and their cell cycle distribution assessed by FACS (fluorescence-activated cell sorting) analysis. The percentage of cells in S-phase ranged from approximately 15% to 40%. Approximately 50 ug of protein was subjected to SDS/PAGE, blotted, and probed with antibodies to cell cycle regulatory proteins. As loading controls, the gels were immunoblotted with antibody to CDK1/CDK2, directed towards the PSTAIRE sequence, as well as an antibody to PCNA, the processivity subunit of DNA polymerase delta, each of which appear to be expressed at relatively constant levels in the cell lines. We initially employed antibodies to 7 cell cycle regulatory proteins, cyclin D1, Rb, cyclin E, CDK2, CDK4 and CDK6. Later the number of cell cycle regulatory proteins examined was expanded to include the tumor suppressor p16, an inhibitor of CDK4/6 which had recently been shown to be deleted or unexpressed in a variety of human tumors (Kamb et al., 1994; Nobori et al., 1994; Herman et al., 1995), as well as the CDK inhibitor proteins p27, p21 and cyclins A, B1, D2 and D3. Representative immunoblots of the more significant components studied are shown in Figure 1.

We observed relatively constant levels of the S-phase and M-phase specific cyclins A and B1. Small differences in the expression of these cyclins between cell lines reflected variations in cell cycle distribution as identified by FACS analysis. Cyclins D2 and D3 were expressed at barely detectable levels in the cell lines examined. The expression of Cyclin E protein appears to be maintained at a relatively high level in most of the breast cancer cell lines as well as in the non-tumor cell line, MCF-12A (Figure 1) (see also Gray-Bablin et al., 1996). In addition, the breast cancer cell lines DU4475 and MBA-MB-157 show unusually high levels of the protein relative to the rest of the panel. We observed marked variations in the expression of both p21 and p27 ranging from a complete lack of expression to overexpression relative to the normal breast cell line, MCF-12A (Figure 1). However, there did not appear to be a correlation between the expression of either of these two proteins and any of the other cell cycle proteins assessed.

Combined overexpression of cyclin D1 and inactivation of p16 appears to be a frequent mechanism for overcoming Rb regulation in breast cancer.

In our initial studies of cell cycle regulatory proteins, we observed a concomitant expression of cyclin D1 and Rb in all but three of the breast cancer cell lines analyzed, COLO 591, ZR75.1 and MDA-MB-175VII. Subsequently, we repeated the analysis of the panel of cell lines for the expression of both Rb and cyclin D1 using three independently isolated protein extracts per cell line. Significantly, both ZR75.1 and MDA-MB-175VII, which initially appeared to lack Rb expression, demonstrated normal levels of Rb protein expression in the newly isolated extracts. Further analysis of the original extracts from these two cell lines confirmed the suggestion that the Rb protein had been degraded by the action of proteases thus giving rise to falsely negative results. Consistent with the original extract, the three new COLO 591 extracts showed a lack of Rb protein expression but virtually undetectable levels of cyclin D1 compared to the moderate levels described in the original report. The signal detected initially using the original cyclin D1 antibody has since been shown in numerous western blots to correspond to the homologous cyclin D3 protein, due to cross reaction with D3 running at a slightly lower molecular weight in the gels (Figure 1). Based upon these revised findings, all 12 of the breast cancer cell lines analyzed (and the normal breast epithelial cell line, MCF-12A as well as HBL-100) fall into two groups: (1), those that express or overexpress cyclin D1 in the presence of Rb, and (2), those that fail to express Rb and also show the absence or very low levels of cyclin D1. Nine of the twelve tumor cell lines showed concomitant expression of cyclin D1 and Rb proteins with six of the nine expressing abnormally elevated levels of cyclin D1 protein relative to that shown by MCF-12A. The degree of over-expression ranged from approximately 4-5-fold (MDA-MB-175VII and MDA-MB-361) to 10-fold (MCF-7, MDA-MB-330, ZR75.1 and MDA-MB-415) as quantitated by densitometric analysis and normalization to the PCNA signal. Three of the twelve tumor cell lines did not express Rb protein and either failed to express or showed barely detectable expression of cyclin D1 protein. This correlation supports previous indications of the dependence of cyclin D1 expression upon the production of functional Rb protein and is illustrated by the HBL-100 cell line, which expresses SV40 large T-antigen, and therefore does not express cyclin D1 due to the inactivation of Rb by the T-antigen.

With the exception of one cell line, MDA-MB-157, p16 protein was not detected in any of the nine cell lines that expressed both Rb and cyclin D1. This contrasts with the cell lines HBL-100 and MDA-MB-231, that lacked both cyclin D1 and Rb expression (or functional Rb in the case of HBL-100), but expressed p16 protein. These data suggest that the combined loss of p16 and overexpression of cyclin D1 is a mechanism frequently employed in breast cancer cell lines to overcome the Rb-mediated G1/S regulation. Since two of the cell lines overexpressing Rb, T47-D and Hs578T, demonstrated only moderate levels of cyclin D1 (approximately equivalent to MCF-12A), it would appear that the absence of p16 expression, in the presence of normal to elevated cyclin D1, is sufficient to overcome Rb activity in a majority of the breast cancer cell lines studied. It should be noted that DU4475 and COLO-591 are unusual in that they fail to express both Rb and p16 proteins. These data confirmed observations made by others of a high frequency of p16 inactivation in breast cancer (Geradts and Wilson, 1996; Xu et al., 1994; Brenner and Aldaz., 1994). In those cell lines lacking Rb protein, the need for cyclin D1 function to

advance cells through G1 into S-phase is obviated (as is the case for DU4475, COLO 591 and MDA-MB-231). The loss of Rb protein therefore appears to provide an alternative mechanism for overcoming G1/S regulation, which is employed in a smaller number of the breast cancer cell lines studied. These findings parallel earlier studies in our laboratories, in which it was found that non-small cell (NSCLC) and small cell (SCLC) lung cancer cells appear to employ similar alternative mechanisms for overcoming cell cycle regulation, with NSCLC cells overexpressing cyclin D1 in the absence of p16, and SCLC cells failing to express Rb. (Schauer et al., 1994)

Expression of Cyclin D1-associated CDK4 and CDK6 proteins in breast cancer cell lines and tumors.

The levels of the Cyclin D1-associated CDK4 and CDK6 proteins remain relatively constant during the normal cell cycle. (Hunter and Pines, 1994). Characterization of these proteins in the breast cancer cell lines involved the use of the specific CDK4 and CDK6 immunizing peptides to block non-specific antibody binding. Three of the eight tumor cell lines, MCF-7, MDA-MB-231 and ZR75.1, and the non-tumor cell line, HBL-100 exhibit elevated levels of CDK4 protein relative to the other tumor cell lines and non-tumor MCF-12A cell line. By contrast, the levels of CDK6 protein do not appear to be significantly increased in the majority of breast cancer cell lines relative to the non-tumor controls (Figure 1). Furthermore two of the breast cancer cell lines lack detectable CDK6 protein. This suggests differences in the potentially oncogenic activities of CDK4 and CDK6 during tumorigenesis in different breast tumors. Previous studies have indicated possible oncogenic roles for CDK4 and CDK6 in tumorigenesis. Tam et al. (1994) described the overexpression of the CDK6 protein in some types of tumor, and amplification of the both the CDK4 gene (Khabtib et al., 1993) and the CDK4 protein (Tam et al., 1994) has been demonstrated in several tumors including breast cancer. Furthermore, a mutated CDK4 protein has been identified in two primary melanoma tumors that prevents binding of the CDK-inhibitor, p16, resulting in constitutively active Cyclin D1/CDK4 complexes (Wolfel et al., 1995). The above data suggest that CDK4 has the potential to contribute to tumorigenesis through at least two pathways: aberrant overexpression or mutation, resulting in each case in escape from p16-mediated cell cycle regulation.

Mechanisms of cyclin D1 protein overexpression in breast cancer.

1) Amplification and elevated transcription of the cyclin D1 gene

Using a 1.1 kb probe from the 5' end of the cyclin D1 cDNA, the panel of breast cancer cell lines were assessed for the copy number of the gene. The MCF-12A cell line was shown to contain two normal copies of chromosome 11 by karyotype analysis (Dr. Leila Garcia, personal communication) and thus was a suitable diploid control for the cyclin D1 locus at 11q13. The cyclin D1-specific hybridization signals in the tumor cell lines were compared directly to that of MCF-12A to determine the copy number of the gene. Visual inspection indicated that all 9 of the cell lines that over-expressed cyclin D1 protein showed amplification of the gene. Densitometric analysis of the cyclin D1 hybridization signals normalized to the B-actin diploid locus, indicated 2-fold amplification in MDA-MB-330 and

MDA-MB-231, 3-fold amplification in MCF-7, MDA-MB-175VII and MDA-MB-361, 5-fold amplification in ZR75.1 and 10-fold amplification in MDA-MB-415. Overall, there was only a moderate correlation between amplification and protein expression. In particular, MDA-MB-231 showed amplification of the cyclin D1 gene but did not show detectable expression of the protein.

Analysis of cyclin D1 transcription also showed significant discrepancies between mRNA levels and protein expression in the cell lines. By comparison to the MCF-12A transcript level and normalization to beta-actin transcript levels, densitometric analysis showed 2-fold cyclin D1 mRNA overexpression in MCF-7, MDA-MB-230, ZR75.1, MDA-MB-175VII and MDA-MB-361, and 5-fold in MDA-MB-415. The data from both the Southern and northern analysis indicate that the mechanism of overexpression of the cyclin D1 protein involves both DNA amplification and increased transcription of the gene. However, when mRNA and protein expression are compared, there are significant discrepancies, in particular in three of the cell lines (MCF-7, MDA-MB-230, ZR75.1), where the level of cyclin D1 overexpression (10-fold) is 5 times greater than would be expected form the observed increases in mRNA levels (2-fold) (Table 2).

2) Post-translational stabilization of the cyclin D1 protein

The discrepancies between increases in cyclin D1 mRNA and protein expression seen in some cell lines indicated that additional mechanisms contribute to the overexpression of cyclin D1. We therefore examined whether there were changes in the half-life of cyclin D1 protein in the cell lines that overexpressed it. Subconfluent cultures of each of the lines were treated with cycloheximide and harvested at given intervals thereafter. With the exception of ZR75.1, all of the cell lines assessed demonstrated the expected cyclin D1 half-life of approximately 20-30 minutes (Figure 2). In ZR75.1 cells, however, cyclin D1 protein levels remained high throughout the time course of the experiments, with approximately 60% of the protein remaining at 150 minutes post cycloheximide treatment. This approximately 5-fold increase in half-life corresponds to the 5-fold greater than expected levels of cyclin D1 protein found in these cells, based on increases in mRNA. This indicates that post-translational stabilization of cyclin D1 protein may be employed by a small proportion of breast cancer cells as a further mechanism for maintaining elevated levels of cyclin D1 protein. Post-translational stabilization of cyclin D1 protein as a mechanism for overexpression has been reported in only one other type of cancer, that of uterine sarcoma cell lines (Weleker et al. (1996).

Expression of cyclin D1 protein in breast cancer cell lines is serum-independent

Another potential mechanism that might contribute to cyclin D1 overexpression is the possibility that the expression of cyclin D1 is no longer regulated by environmental growth factors and serum in breast cancer cells. To address this possibility, we cultured all nine of the breast tumor cell lines that expressed moderate to elevated levels of cyclin D1 and the normal breast epithelial cell line, MCF-12A, in the absence of serum for one, two or three days respectively. Following protein extraction, the cell lines were analyzed for the expression of cyclin D1 by immunoblot analysis. Unlike normal macrophage cells that fail

to sustain expression of cyclin D1 in the absence of serum (Hunter and Pines., 1994), none of the breast cancer cell lines nor the immortalized non-tumor breast epithelial cell line MCF-12A, demonstrated a decrease in cyclin D1 protein expression in response to serum deprivation (Figure 3). The absence of a requirement for mitogenic stimulation of cyclin D1 expression may therefore represent a step toward cellular immortalization and transformation by removing a regulatory barrier to cyclin D1 expression and the subsequent uncontrolled progression through G1 into S-phase.

Expression of cyclin D1 protein in breast cancer cell lines is largely independent of cell contact-mediated negative regulation.

The apparent independence of cyclin D1 expression from external growth cues in the breast cancer cell lines was further investigated by assessing the effect of cellular confluence upon its expression. Three of the breast cancer cell lines that showed 10-fold overexpression of cyclin D1 (MCF-7, ZR75.1 and MDA-MB-330) and the normal epithelial cell line, MCF-12A, were cultured to different degrees of confluency: 50% confluency (cells in logarithmic phase of growth), 85% confluency, and 100% confluency. Extracts prepared from each set of culture conditions were analyzed for the expression of cyclin D1 protein by immunoblot analysis. The normal MCF-12A cell line showed a reduction in the expression of cyclin D1 to barely detectable levels once the cells reached 100% confluence, indicating that contact inhibition caused an essentially complete repression of cyclin D1 expression (Figure 4). In contrast, the breast cancer cell line ZR75.1 showed no reduction in cyclin expression upon reaching confluence (Figure 4), while MCF-7 and MDA-MB-330 cells exhibited 50% or less reduction (data not shown), such that cyclin D1 was still markedly overexpressed in these cells at confluence compared to normal MCF-12A cells growing logarithmically. The lack of substantial reduction in the level of cyclin D1 in the confluent breast cancer cell lines is most likely attributable to a loss of normal cell contact-mediated negative regulation of cyclin D1 expression in these cells.

Mechanisms of p16 inactivation in breast cancer

<u>1) Homozygous deletion of the p16 gene is a frequent mechanism of inactivation in breast cancer.</u>

Since ten of the twelve tumor cell lines did not express p16 protein, we assayed the cell lines for the homozygous deletion of the p16 locus at chromosome 9p21. The cell lines MDA-MB-231, MDA-MB-157 and HBL-100 as well as an additional breast cancer cell line that expressed p16 were included as positive controls for p16 DNA. This was achieved by subjecting the cell line DNA to multiplex PCR analysis using oligonucleotide primers designed from intron 2 of the p16 gene in combination with primers from the D9S199 locus. Since the D9S199 locus is localized distal to the p16 gene at 9p23 and therefore unlikely to be within the homozygously deleted region on chromosome 9p, it was employed as a positive control for PCR amplification. The two normal breast epithelial cell lines, MCF-12A and HBL-100, and six of the ten tumor cell lines (that did not express p16 protein) successfully amplified both the p16 and D9S199 amplicons. However four tumor cell lines showed

D9S199 amplification but failed to amplify the p16 product (Figure 5). Thus, the homozygous loss of the p16 locus accounted for the lack of p16 protein expression in 4 of the 10 cell lines studied. The remaining 60% of cases may therefore result from mechanisms including mutation of the gene or alterations in transcription induced by, for example, methylation.

2) The p16 gene is methylated in 30 percent of breast cancer cell lines

Since six of the ten breast cancer cell lines that did not express p16 protein contained the p16 gene, we assessed these cell lines for methylation of the p16 gene. Cell line DNA was modified overnight using sodium bisulfite to convert all of the unmethylated cytosines to uracil. The resulting DNA was then subjected to PCR analysis using oligonucleotide primers designed from the promoter of the p16 gene that were specific for wild type (unmodified), methylated and unmethylated DNA (Herman et al., 1996). Three of the four cell lines that were previously shown to have undergone the homozygous deletion of the p16 gene (COLO 591, MCF-7, Hs578T) were included as negative controls for amplification. Two of the cell lines, DU4475 and T-47D demonstrated amplification using the methylated-specific oligonucleotides only, while a third cell line, ZR75.1 showed amplification using both methylated- and unmethylated-specific oligonucleotides (Figure 6). The latter result indicates either that the p16 promoter is hemi-methylated in ZR75.1 or that ZR75.1 is composed of a heterogeneous cellular population. The remaining three cell lines that lacked p16 expression but were not homozygously deleted for the p16 gene, underwent amplification with the oligonucleotides specific for unmethylated DNA only. Thus, 7 of the 10 cell lines that did not express the p16 protein had undergone either the homozygous deletion or methylation of the gene. The status of the p16 gene in the cell lines studied is summarized in Table 3. These results concerning homozygous deletion and methylation of p16 genes are consistent with the findings of others in various types of human cancer (Kamb et al., 1994; Nobori et al., 1994; Liu et al., 1995; Herman et al., 1996; Cairns et al., 1994). The remaining 30 percent of cell lines lacking p16 protein are likely to have undergone mutation of the p16 gene and deletion of the remaining wildtype allele (loss of heterozygosity). However, in the absence of highly polymorphic markers within the p16 gene and the corresponding normal cell line counterpart to each of the remaining three cell lines, it is not possible to assess them for loss of heterozygosity at the p16 locus.

Analysis of cyclin D1 complexes in breast cancer cell lines.

Our immunoblot analysis of breast cancer cell lines indicated that breast cancer cells most frequently overcome Rb-mediated G1/S regulatory control by loss of p16 expression coupled with moderate or elevated expression of cyclin D1. To determine whether breast cancer cells that exhibit this type of cell cycle regulatory defect produce constitutively active cyclin D1/CDK4/CDK6 complexes and therefore have the potential to inactivate Rb, we have analyzed kinase activity in cyclin D1/CDK4/CDK6 complexes using a glutathione S-transferase-Rb-(C) fusion protein as substrate (gift from Dr. J. Gregori, Dept. of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center). The fusion protein consists of the C-terminus of Rb fused to GST resulting in a molecule of 45

Kd. Following purification of the GST-Rb-(C) protein (described in Materials and Methods), aliquots of each of the four sequential elution products were analyzed for concentration and purity by Coomassie blue staining of electrophoretically separated proteins on a SDS-polyacrylamide gel in comparison to carbonic anhydrase protein standards of known concentration.

We choose the breast cancer cell line, MCF-7, for the analysis of cyclin D1 complex kinase activity since it expressed Rb, showed 10-fold overexpression of cyclin D1 relative to the normal breast epithelial cell line, MCF-12A, and lacked p16 expression. The obligate CDK partners of cyclin D1, CDK4 and CDK6, were expressed at very low and moderate levels, respectively, in this cell line (Figure 1). Lysates of MCF-7 were prepared as described in Materials and Methods and immunoprecipitated with antibodies to cyclin D1, CDK4 and CDK6 (all of which are components of cyclin D1 complexes in mid G1 phase), CDK2 (which is associated with cyclin E and cyclin A complexes in late G1 and early S-phase respectively), and normal rabbit serum (as a negative control). The immunoprecipitates were then assayed for kinase activity using GST-Rb-(C) as the substrate (Figure 7). Our analysis showed a high level of kinase activity in the CDK2 immunoprecipitate but a very low level of activity in the cyclin D1, CDK4 and CDK6 immunoprecipitates. Low levels of kinase activity in cyclin D1 and CDK4 immunoprecipitates from cyclin D1-overexpressing non-small cell lung cancer cell lines have been observed by Grimison and Sclafani (unpublished results), suggesting that the currently available antibodies for cyclin D1 and CDK4, unlike those for CDK2, are not optimal for the detection of kinase activity despite their usefulness in immunoblot analysis. Although a low level of CDK4-associated kinase activity in MCF-7 cells is to be expected from the low level of CDK4 protein detected in these cells, there are substantial levels of CDK6 protein present, and it seems unlikely that rapidly growing cells expressing Rb would have low levels of all forms of cyclin D1-activated kinase activity. We therefore investigated whether the low level of CDK6-associated kinase activity found is due to technical problems associated with the optimization of immunoprecipitate-kinase assays, to the choice of antibodies, or perhaps to a combination of these factors. However, these attempts to overcome the technical or other problems associated with detection of cyclin D1/CDK6 kinase activity did not succeed, and therefore did not allow any conclusions with regard to the existence of constitutively active forms of this kinase in breast cancer cells lacking p16 and expressing cyclin D1.

Expression of cell cycle regulatory proteins in breast tumor tissues.

Studies of the characteristics of tumor-derived cell lines which have necessarily been subjected to selection for growth and long-term maintenance in culture have the important disadvantage that these cells may exhibit defects in growth regulation that do not reflect the state of the original tumor tissue. We therefore examined a panel of ten matched pairs of normal and breast tumor tissues, provided to us by Dr. Wilbur Franklin from the Colorado Cancer Center tissue procurement core. Western blot analysis and comparison of the ten pairs of normal and tumor tissues indicated that breast tumors also display the same type of cell cycle regulatory defects found in breast cancer cell lines. Eight of ten tumors showed expression of cyclin D1 in the presence of Rb, while the remaining two failed to express Rb

and contained undetectable or barely detectable levels of cyclin D1. Of the eight tumors that expressed Rb, six showed high or very high overexpression of cyclin D1 and two showed cyclin D1 levels comparable to normal breast tissue. (Figure 8 and Table 4). We then addressed the question of whether the high level of cyclin D1 protein expressed by most of the tumors was an event involved in tumorigenesis or merely a reflection of their greater proliferative rate compared to the corresponding normal tissues. The western blots previously probed with the cyclin D1 antibody were stripped and reprobed with anti-CDK1/CDK2 antibody, directed towards the PSTAIRE epitope (Figure 8). The tumor COBRC4(T,) which expressed only barely detectable levels of cyclin D1 protein, showed a high level of CDK1/CDK2 expression whereas two of the three tumors that overexpressed cyclin D1, did not express elevated levels of PSTAIRE protein. The fourth tumor, COBRC2(T), shows elevated levels of both cyclin D1 and PSTAIRE proteins. These data indicate that the expression of cyclin D1 in the tumors is not correlated simply with cellular proliferation rate but is associated with the occurrence of breast malignancy.

In these studies, the p16 protein was not detected in any of the normal or tumor tissue samples assessed by western blot analysis. This reflects similar difficulties in other laboratories. It has been demonstrated that p16 protein is more difficult to detect than Rb protein in a variety of normal tissue type, particularly in breast tissue. (Geradts and Wilson, 1996). It is therefore likely that the western blot procedure is insufficiently sensitive for the detection of p16 protein expression in tissue extracts. However, more recently we have obtained an antibody which is capable of readily detecting p16 protein in breast tissues by immunoblotting, and have used this antibody to further assess the nature of the cell cycle regulatory defects occurring in breast tumors and cell lines (see Section III and IV below).

RESULTS - SECTION II

ALTERATION OF THE TRANSFORMED PHENOTYPE OF BREAST CANCER CELLS BY TRANSFECTION OF CELL CYCLE REGULATORY CONSTRUCTS

Construction of antisense cyclin D1 adenoviral vectors.

The work described above has shown that essentially all breast cancer cells have defects in the expression of cell cycle regulatory components that act at the G1/S boundary. The most frequent pattern of defects observed is the overexpression of cyclin D1 in the presence of Rb, coupled with the absence of p16 expression. We therefore sought to directly determine whether correction of these defects by transfection of appropriate expression constructs would alter the transformed characteristics of breast cancer cells. Since this work was initially proposed, three groups have examined the effects of correcting either abnormal cyclin D1 overexpression or lack of p16 expression in other types of malignancies. Following transfection with antisense cyclin D1 both a human esophageal cell line and a mouse lung cancer cell line were suppressed in their ability to induce tumor formation in nude mice (Zhou et al. (1995); Schrump et al. (1995)). Jin et al. (1995) have also shown that the introduction of p16 into non-small cell lung cancer cell lines lacking p16 expression using a recombinant adenoviral vector greatly reduces their ability to induce tumors in nude mice. These data indicate that the overexpression of cyclin D1 and/or inactivation of p16 do indeed play a role in the development of human malignancy and that the correction of just one of these abnormalities is sufficient to induce reversion to the non-transformed state. We initially proposed to clone antisense cyclin D1 into the pRc/RSV and pRc/CMV mammalian expression vectors (Invitrogen). However, owing to more recent studies that have demonstrated highly efficient transformation of mammalian cell lines using adenovirus-based vectors compared to both retroviral and plasmid vectors (Badie et al., 1994; Liu et al., 1995; Qian et al., 1995), we choose instead to use the recombinant adenoviral vector, 327 Bst B-Gal (gift from Dr. Jerome Schaack, Department of Microbiology, University of Colorado Health Sciences Center), for the introduction of antisense cyclin D1 into breast cancer cell lines. Candidate breast cancer cell lines for these experiments were chosen based upon the fact that they demonstrated (a) over-expression of cyclin D1 protein and a lack of p16 protein expression; and (b) tumorigenicity in nude mice.

1) Assessment of breast cancer cell line tumorigenicity.

Six of the twelve breast cancer cell lines in the panel showed over-expression of cyclin D1 protein but the majority of these cell lines had been previously shown to be non-tumorigenic in nude mice (Caileau et al., 1974; Hackett et al., 1977; Cailleau et al., 1980). However, there was contradictory information or lack of information regarding the tumorigenicity of MCF-7 and ZR75.1, both of which showed over-expression of cyclin D1 protein and no p16 expression. MDA-MB-231 had been previously shown to be tumorigenic in nude mice (Price et al., 1990). To determine the tumorigenicity of MCF-7 and ZR75.1,

each cell line was injected at 5 X 10^6 cells subcutaneously into the right shoulder of three male athymic nude mice. We also injected 5 x 10^6 MDA-MB-231 cells into three mice as positive controls. Tumor dimensions were measured bi-weekly for 28 days at which time the mice were euthanised and the tumors resected and measured. The three mice injected with MCF-7 cells showed either no tumor growth or growth of very small tumors. However, all three of the mice injected with the ZR75.1 and MDA-MB-231 cell lines developed tumors at the injection site ranging in size from 0.5 to 2.0 cm (Table 5). The small size and unreliable occurrence of the tumors formed by MCF-7 cells made these cells appear to be a poor choice for testing of effects of cell cycle regulatory protein expression on tumorigenicity. However, we subsequently learned that MCF-7 cells reliably form tumors of good size in mice supplied with estrogen (see below). Therefore we proceeded to use MCF-7 and ZR75.1 as test cell lines for the introduction of constructs that alter cell cycle regulatory protein expression.

2) Characteristics of the Adenoviral vector 327_{Bst}B-gal.

The vector 327_{Bst}B-gal is a recombinant adenovirus serotype 5. The vector lacks the E1 region (containing the E1a and E1b genes) which is essential for replication, and contains a partially disrupted E3 region, which appears to be involved in the host cell immune response. The E1 region has been replaced by the E. coli B-Galactosidase gene driven by the CMV immediate early promoter, and a unique BstBI site has been inserted 3' to the B-Galactosidase gene. As a result the vector is only capable of replicating in the Ad5-transformed cell line 293, a human kidney embryonal cell line that expresses both E1a and E1b. To construct a recombinant adenovirus expressing a given gene, the latter is first sub-cloned into a plasmid vector PACCMVpLpA (gift from Dr. Jerome Schaack, Department of Microbiology, University of Colorado Health Sciences Center) under the control of a CMV promoter. The plasmid contains sequences homologous to the left end of Ad5, such that following co-transfection of 293 cells with both linearized PACCMVpLpA and 327_{Bst}B-gal digested with BstBI followed by X-Gal staining, overlap recombination between the plasmid and virus results in clear plaques (which may then be isolated) whereas mere religation of the viral arms results in blue plaques.

3) Determination of the efficiency of 327_{Bst}B-gal infection of ZR75.1 cells.

Following the calculation of the viral titer at 108 pfu/ml and isolation of 327_{Bst}B-gal DNA (see Materials and Methods), we determined the concentration of virus necessary to infect the ZR75.1 cells at a high efficiency without accompanying cytopathic effects upon the cells. Thus, we infected ZR75.1 cells with 10^5, 10^3 and 10 pfu/ml of the viral stock and after 24 and 48 hours respectively, stained the cells with X-Gal to determine the proportion expressing B-Galactosidase (as judged by a blue coloration). After 24 hours approximately 70-80% of the cells infected with 10^5 pfu/ml and 60% infected with 10^3 pfu/ml were blue. By 48 hours post-infection, 100% of the cells infected with 10^5 pfu/ml and approximately 80% infected with 10^3 pfu/ml were blue. Further, the proportion of blue cells in the plates infected with 10 pfu/ml was approximately 30%. However, there was an associated increase in the cytopathic effect after 48 hours in the cells infected with 10^3 and 10^5 pfu/ml viral stock. We therefore concluded that the optimal concentration for infection was between 10^3

and 10^4 pfu/ml followed by removal of the virus-containing media after 18 hours.

4) Immunogenicity of 327_{Bst}B-gal in nude mice.

Previous reports have indicated the immunogenic effect of adenoviral vectors upon mammalia hosts (Schaack et al., 1995). We therefore assessed the effect of 327_{Bst}B-gal-infected ZR75.1 cells upon nude mice in order to ensure that future tumorigenicity assays would not be compromised by a host inflammatory response. Although the adenoviral E3 gene had been partially disrupted in this vector, it was not known if the remaining gene product was sufficient to elicit an immune response. ZR75.1 cells were infected with 10^4, 10^6 and 10^7 pfu/ml viral stock (equal to and greater than that would be used for the transfection assays) and at 24 hours post-infection, harvested and injected in duplicate at 5 x 10^6 cells subcutaneously into male athymice nude mice. As a control two mice were also injected with non-infected ZR75.1 cells. The mice were monitored bi-weekly for signs of inflammation at the injection site and for tumor development. After 28 days, the mice were euthanised, examined both externally and internally at the injection site and the tumors resected. None of the mice showed evidence of inflammation. Furthermore, the sizes of the tumors that grew in the mice injected with or without 327_{Bst}B-gal-infected ZR75.1 cells were equivalent indicating that the viral vector itself does not suppress the tumorigenicity of the ZR75.1 cells.

5) Construction of a PACCMVpLpA-antisense cyclin D1 vector.

A 1.1 kb HindIII-XbaI fragment from the 5' end of the cyclin D1 cDNA (Invitrogen) was subcloned in the antisense orientation into the XbaI-HindIII site of PACCMVpLpA. Initially, the ligations were transformed into DH5-alpha cells. However, the resulting clones were all highly rearranged indicating a high degree of recombination in this particular bacterial host. We therefore chose to use the rec BC^- strain, SURE (Stratagene), from which we obtained stable PACCMVpLpA-antisense cyclin D1 transformants.

6) Co-transfection of PACCMVpLpA-antisense cyclin D1 constructs with 327_{Bst}B-gal adenoviral vector DNA into 293 cells.

Five micrograms of each of two stable PACCMVpLpA-antisense cyclin D1 constructs were linearized with ClaI and two micrograms of the CsCl-purified 327_{Bst}B-Gal DNA were digested with BstBI to cut 3' to the beta-Galactosidase gene. We then individually co-transfected 5 micrograms of each of the plasmid construct DNA digests with one microgram of the adenoviral vector DNA into kidney 293 cells by calcium phosphate precipitation. However, in spite of very extensive attempts, repeated employment of this procedure failed to produce recombinants between 327_{Bst} B-gal and the PACCMVpLpA plasmid constructs. Thus, in spite if the large amount of effort expended on the development of adenoviral vectors for the introduction of cell cycle regulatory proteins, we were forced to abandon this approach.

Construction of sense p16 and antisense cyclin D1 Tet-regulated plasmid vectors.

On abandonment of the adenoviral vector approach, we initially planned to return to the use of the plasmid constructs described in our original proposal to transfect breast cancer cells. However, at that time the Tet expression system became readily available to us as a result of ongoing studies in our laboratories on the effects of p16 and antisense cyclin D1 on lung cancer cell proliferation. The Tet expression system (Gossen et al., 1995) enables the highly regulated expression of genes in response to tetracycline or its derivative, doxycycline. We have chosen to use the Tet-Off system whereby gene expression (either in the sense or antisense orientation) is turned off in the presence of doxycycline to introduce either sense p16 or antisense cyclin D1 into the breast cancer cell lines MCF-7 and ZR75.1. Following stable transfection of the cell lines with constructs containing either p16 or antisense cyclin D1, the expression of the transfected gene may be regulated by the addition or withholding of doxycycline in the culture medium.

However, prior to initiating studies of the effects of Tet-regulated p16 expression, we tested MCF-7 and ZR75.1 cells for sensitivity to the expression of functional p16 protein by infecting them with a newly available p16-expressing adenoviral construct, Ad-P16 (Invitrogen) that constitutively expresses p16 under the control of a CMV promoter (Schrump et al., 1996).

1) Sensitivity of MCF-7 and ZR75.1 cells to expression of p16 protein

Following the growth of MCF-7 and ZR75.1 cells to approximately 50% confluence in 100 mm plates, they were infected with Ad-p16 at multiplicities of infection (moi) of 10, 50 and 100, and incubated at 37°C for 42 hours. Control cells were left uninfected. After incubation, the uninfected and Ad-p16-infected cells were harvested both for FACS analysis to determine cell cycle distribution and immunoblot analysis to determine the expression profiles of cell cycle regulatory proteins. Both uninfected MCF-7 and ZR75.1 cells showed the expected cell cycle distribution and regulatory protein expression profiles for logarithmic cellular populations and lacked p16 expression. Infection with Ad-p16 resulted in expression of very high levels of p16, together with nearly complete G1 arrest and cessation of growth. Representative data for MCF-7 cells are shown in Figure 9. The effect of p16 expression upon the cell cycle was also illustrated by the expression profiles of cell cycle regulatory proteins. Rb protein was completely shifted to the fully active hypophosphorylated state, as shown by the single band of high gel mobility, in keeping with a complete inhibition of CDK4/6 cyclin D1 kinase activity. Cyclin D1 itself showed decreasing expression with increasing levels of p16. This implies that the binding of p16 to CDK4/6 proteins resulted in the rapid degradation of monomeric cyclin D1 in those cells infected with Ad-p16 at high moi. In addition, cyclin A and B proteins, which are expressed on entry into S- and M-phase, respectively, were completely lacking in Ad-p16-infected cells (Figure 9). The complete cessation of cell growth caused by Ad-p16 infection indicated that p16 was expressed at essentially toxic levels in these cells. However, these experiments demonstrated that both MCF-7 and ZR75.1 cells were sensitive to p16 function, and therefore suitable cell lines for the analysis of the effects of regulated p16 expression upon the transformed properties of

breast cancer cell lines. We therefore proceeded with the construction of Tet-regulated vectors and transfectants for the expression of p16 as well as antisense cyclin D1.

2) Characteristics of the Tet expression system

The two plasmid vector components of the Tet system were kindly provided by Dr. Bujard, Heidelberg, Germany. The gene of interest is first cloned into a "response" plasmid, pTET-SPLICE, under the control of the Tet Operator (tet O) from the tet resistance operon of E. coli Tn 10, and the minimal immediate early promoter of CMV. In E. coli, the tet repressor protein (tet R) tightly binds the tet O except in the presence of tetracycline or doxycycline. A second "regulator" plasmid, pUDH15-1, expresses a hybrid protein called the tet-controlled transcriptional activator (tTA) which consists of the tet repressor DNA binding domain fused to the VP16 activation domain of herpes simplex virus. tTA binds the tet O sequence, and activates transcription of the target genes in the absence of tetracycline or doxycycline.

2) p16 and antisense cyclin D1 Tet constructs

A functional 0.5 Kb BamHI-XbaI fragment from the 5' end of the p16 gene (gift from Dr. A. Kamb, Myriad Genetics, Utah) was cloned into the HindIII-SpeI sites of the "response" plasmid, pTET-SPLICE in the sense orientation; and a 1.1 kb cyclin D1 fragment was cloned into the HindIII-SpeI sites of the "response" plasmid, pTET-SPLICE in the antisense orientation, by Dr. M. Dalton in the Sclafani laboratory. The "regulator" plasmid, pUDH15-1, was modified by the cloning of the neomycin-resistance gene into the XhoI site site to enable G418 selection.

3) Optimization of lipid-mediated transfection

We chose the highly efficient lipid-mediated transfection method to introduce the Tet system constructs into our breast cancer cell lines. Initially we tested a panel of 8 different lipids (Invitrogen) for their ability to transfect the pCMV-Luc plasmid (containing the luciferase gene under a constitutive promoter) into the MCF-7 and ZR75.1 cell lines. The relative transfection efficiencies mediated by each lipid were judged by assay of luciferase expression after incubation for 24 hours at 37°C (see Materials and Methods). Of the eight lipids, several showed 10^4 to 10^5-fold greater luciferase activity than the non-transfected control. The highest level of activity was demonstrated by cells transfected using number 7. This particular lipid was therefore chosen for all further transfection experiments.

Transient transfection of MCF-7 and ZR75.1 with sense p16 and antisense cyclin D1 constructs

To ensure that the tet system vector constructs were functioning correctly in our breast cancer cell lines prior to producing stable transfectants, we initially performed transient transfections of MCF-7 and ZR75.1 with the sense p16 and antisense cyclin D1 constructs.

1) p16 transient transfections

The transfection assays were performed on MCF-7 and ZR75.1 cells in 6-well plates, during logarithmic growth. The cells were transfected with (a) 2.4 ug/well of both the pTET-SPLICE-p16 (response plasmid) and the pUDH15-1 (regulatory plasmid); (b) 2.4 ug/well of the pTET-SPLICE-p16 plasmid, as a negative control; and (c) 2.4 ug/well of the pCMV-Luc plasmid, as a control for the lipid-mediated transfection efficiency. Cells were also left untransfected as a further negative control. After 5 hours at 37°C the DNA/lipid mixture was aspirated off the cells and replaced with medium containing 10% serum and 1ug/ml doxycycline. Following 24 hours at 37°C, the appropriate cells were harvested and assessed for p16 and luciferase expression. The lipid-mediated transfection efficiency was shown to be high based upon analysis of luciferase expression in the pCMV-Luc transfected cells. This implied that the Tet system plasmids should also have been transfected at a high efficiency. Immunoblot analysis revealed that in those cells transfected with both pTET-SPLICE-p16 and pUDH15-1, the expression of p16 was "on" in the absence of doxycycline and "off" in the presence of doxycyline (Figure 10). These data confirm that the regulation of p16 expression by doxycycline worked successfully in each of the two breast cancer cell lines. Further, those cells either transfected with pTET-SPLICE-p16 alone, or left untransfected, showed no expression of p16 in the presence or absence of doxycycline indicating the absolute requirement for the tTA regulatory protein encoded by pUDH15-1 to allow p16 expression (Figure 10).

2) Antisense cyclin D1 transient transfections

The transient transfection of MCF-7 and ZR75.1 cell lines with the Tet system antisense cyclin D1 construct was performed as described above for the Tet system sense p16 construct. Whereas in the p16 experiment our aim was turn "on" the expression of the gene and thus express the protein, the goal of the antisense cyclin D1 experiment was to turn "on" the expression of the antisense gene, and thus suppress the expression of the cyclin D1 protein. Logarithmic phase cells were transfected with (a) 2.4 ug/well of both the pTET-SPLICE-antisense cyclin D1 (response plasmid) and the pUDH15-1 (regulatory plasmid); (b) 2.4 ug/well of the pTET-SPLICE-antisense cyclin D1 plasmid, as a negative control; and (c) 2.4 ug/well of the pCMV-Luc plasmid, as a control for the lipid-mediated transfection efficiency. Cells were also left untransfected as a further negative control. After 5 hours at 37°C the DNA/lipid mixture was aspirated off the cells and replaced with medium containing 10% serum plus or minus 1 ug/ml doxycycline. Following the growth of cells for 24 hours, they were harvested and assayed either for luciferase or cyclin D1 expression by immunoblot analysis. The degree of luciferase expression in those cells transfected with pCMV-Luc indicated that the transfection efficiency was as high as that observed in the previous p16 transfection experiment. However, we did not observe the expected suppression of cyclin D1 in those cells cotransfected with three different amounts of the pTET-SPLICE-antisense cyclin D1 and pUDH15-1 plasmids, in the absence of doxycycline. In spite of this negative result, we chose to proceed with the construction of stable transfectants of antisense cyclin D1, since the possibility existed that expression levels in stable transfectants would be higher and also be sustained longer, allowing significant reduction of cyclin D1 expression.

Construction of stable p16 and antisense cyclin D1 transfectants

1) Stable transfection of MCF-7 and ZR75.1 with the "regulator" plasmid, pUDH15-1

The production of a "double stable" cell line, in which both the regulator plasmid (pUDH15-1) and the response plasmids (pTET-SPLICE-p16 or -antisense cyclin D1) are integrated in the genome, requires two independent transfection experiments, the first involving the stable integration of the regulator plasmid into each of the cell lines. As mentioned earlier, we cloned the neomycin-resistance gene into the pUDH15-1 plasmid to allow selection for those cells with resistance to G418. Prior to transfecting MCF-7 and ZR75.1 with pUDH15-1, we performed G418 "kill-curve" assays on the two cell lines with concentrations ranging from 300 to 1000 ug/ml. The minimum concentration required to kill all untransfected cells within one week was determined to be 500 ug/ml. Two 6-well plates containing MCF-7 and ZR75.1 cells were grown to 50-60% confluence and transfected with 2.4 ug/well of the pUDH15-1 plasmid encoding the tTA. After 5 hours at 37°C, the lipid/DNA mixture was removed from the cells and replaced with medium containing 10% serum. The cells were grown for a further 48 hours at 37°C before each well was harvested and the cells transferred to one 100 mm dish containing medium supplemented with 10% serum and 500 ug/ml G418. Every 5-7 days, the medium was changed and after two weeks each dish contained approximately 15-20 discrete colonies. As predicted the untransfected cells lacking G418 resistance were killed. A total of 56 G418-resistant colonies from the MCF-7 transfection and 40 colonies from the ZR75.1 transfection were then isolated by "ring cloning". Cells were transferred to 25 cm^2 flasks and at approximately 50% confluence, the cells were harvested for DNA isolation and preparation of frozen stocks, leaving the remainder to grow in culture. The DNA was subjected to PCR analysis using primers that we designed to the tTA sequence (see Materials and Methods) in order to identify those that contained the pUDH15-1 plasmid. Of the 56 MCF-7 clones assessed by PCR, 20 contained the integrated plasmid, and of the 40 ZR75.1 clones assessed, 6 contained the plasmid.

2) Functional analysis of "regulator" pUDH15-1 transfectants

To ascertain if the pUDH15-1 plasmid was functional in those clones that were shown to contain it by PCR, we transiently transfected pTET-SPLICE-p16 into 6 representative MCF-7 and ZR75.1 clones by lipid-mediated transfection. Each of the clones were grown to approximately 50-60% confluence in each of four 35 mm wells, at which time two of the four wells were transfected with 2.4 ug/well pTET-SPLICE-p16, and two were left as untransfected controls. After 5 hours at 37°C the lipid/DNA mixture was aspirated off the cells. To two of the wells containing transfected and untransfected cells, respectively, we added medium containing 10% serum and 500 ug/ml G418 (to maintain the presence of the pUDH15-1 plasmid) plus 1 ug/ml doxycycline (to suppress tTA activation of p16 expression) To the remaining two pairs, the identical medium but lacking doxycycline (to allow tTA activation of p16 expression) was added. Following 24 hours at 37°C, the cells were harvested for p16 expression by immunoblot analysis. Not surprisingly, we observed clonal variation with regard to the degree of p16 induction ranging from none at all, to very strong induction resulting in levels of p16 expression equivalent to or greater than that

shown by the p16 overexpressing breast cell line, HBL-100. In the presence of doxycycline, however, there was no expression of p16, indicating the tightly-regulated, doxycycline-dependence of the system. Of the 6 MCF-7 clones assessed, 4 showed p16 induction, and of the 6 ZR75.1 clones assessed, 3 showed p16 induction. Representative data from MCF-7 and ZR75.1 clones expressing high, moderate and undetectable levels of p16 are shown in Figure 11. Variation in the level of p16 expression likely resulted from differences in the copy number and site/orientation of integration of the pUDH15-1 plasmid in the various independent clones analyzed. Based upon the results of the transient transfections, we chose one MCF-7 and one ZR75.1 transfectant to stably transfect with the pTET-SPLICE-p16 plasmid. Clones MCF-7/15-1#11 and ZR75.1/15-1#3 which exhibited a moderate degree of p16 induction relative to HBL-100, were chosen because our objective was to express a level of p16 that would be sufficiently high to study its effect upon the transformed and tumorigenic properties of breast cancer cell lines, but not so great as to cause cells to completely arrest in G1 (as we observed following infection of cells with the Ad-p16 adenoviral construct).

3) Stable transfection of MCF-7/15-1 and ZR75.1/15-1 with pTET-SPLICE-p16

Because the pTET-SPLICE-p16 construct did not harbor antibiotic resistance, we chose to cotransfect it with the plasmid, pZeo (Invitrogen) which enable the selection of clones based upon resistance to zeocin. Prior to the transfection, we performed zeocin "kill-curve" assays on the two clones with concentrations ranging from 200 to 1000 ug/ml. The minimum concentration required to kill all untransfected cells within 8-10 days was determined to be 500 ug/ml. The MCF-7/15-1 and ZR75.1/15-1 cells were grown in 8 3 mm plates until 50-60% confluence. At this time, 6 of the 8 wells were cotransfected with 2.4 ug/well pTET-SPLICE-p16 and 0.12 ug/well pZeo, and the remaining 2 wells were left untransfected. After 5 hours at 37°C, the lipid/DNA mixture was removed from the cells. To 5 of the 6 wells containing transfected cells, medium containing 10% serum, 500 ug/ml G418 and 1 ug/ml doxycycline (to suppress p16 expression) was added, and to the remaining 3 wells, medium containing 10% serum, 500 ug/ml G418 but lacking doxycycline (to allow p16 expression) was added. The cells were grown for another 48 hours at 37°C before each well was harvested and the cells transferred to each of 8 100 mm plates. The 5 plates containing transfected cells that had been grown in the presence of doxycycline, were cultured in medium supplemented with serum, 500 ug/ml G418, 500 ug/ml zeocin and 1 ug/ml doxycycline, whereas the untransfected cells and the transfected cells previously grown in the absence of doxycycline were cultured in medium identical to that described above but lacking in doxycycline.

4) Isolation of MCF-7/15-1 p16 transfectants

At 7 days post-transfection, all 5 of the 100 mm dishes containing cells cultured in the presence of doxycycline were approximately 40-50% confluent and were therefore replated at lower densities to enable the isolation of discrete independent colonies. By contrast, those transfectants cultured in the absence of doxycycline failed to proliferate and remained non-dividing for the duration of the experiment. We attribute the arrest and eventual senescence of these cells to the lack of doxycycline-mediated suppression of p16

expression. As expected, the two plates containing the untransfected cells were killed in the presence of zeocin.

At 3-5 weeks post-transfection, a total of 94 colonies were isolated by "ring cloning" and transferred to 25 cm^2 flasks. At approximately 50-60% confluence, the cells were harvested for DNA isolation and preparation of frozen stocks, leaving the remainder to grow in culture. Because the p16 gene is homozygously deleted in the MCF-7 cell line, we were able to identify those clones that contained the pTET-SPLICE-p16 plasmid by subjecting the DNA samples to PCR using primers designed to amplify exon 2 of the p16 gene (Hussussian et al., 1994). Of the 94 clones screened by PCR, 29 showed amplification of p16.

5) Isolation of ZR75.1/15-1 p16 transfectants

Unlike the MCF-7/15-1 transfection experiment, at 7 days post-transfection the ZR75.1/15-1 transfectants cultured in the presence and absence of doxycycline appeared unhealthy with a significantly suppressed growth rate. In an attempt to improve the yield of transfectants, we attempted to optimize transfection efficiency by varying the amounts and ratios of pTET-SPLICE-p16 and pZeo DNA used, and altering the concentration of zeocin used for the selection of clones. Manipulation of latter variable proved to be critical in the successful isolation of healthy ZR75.1/15-1 transfectants. Briefly, the transfection procedure was repeated exactly as described above with the exception that the cells were transferred into 100 mm plates containing medium supplemented with 200 ug/ml zeocin instead of 500 ug/ml zeocin at 48 hours post-transfection. After 5 days in the less stringent antibiotic selection conditions, the concentration of zeocin was increased to 500 ug/ml used in the MCF-7/15-1 experiments. This proved a more successful strategy, and resulted in the isolation of 27 zeocin-resistant colonies. DNA was not prepared from these clones since the parent cell line, ZR75.1, contains at least one copy of the p16 gene in its methylated non-transcribed state that would be detected in all of the clones by the exon 2-specific oligonucleotides. We conclude that the initial selection of the ZR75.1/15-1 p16 transfectants in the lower zeocin concentration proved successful as it enabled cells to grow under less stringent conditions until the expression of zeocin resistance by the integrated pZeo plasmid was complete.

5) Analysis of MCF-7/15-1 and ZR75.1/15-1 transfectants for p16 expression

All 29 of the zeocin-resistant MCF-7/15-11 clones that demonstrated p16 amplification, and 22 of the 27 zeocin-resistant ZR75.1/15-1 clones, were assessed for doxycycline-dependent expression of p16 protein. Each of the clones were grown in two 35 mm wells either in the presence or absence of doxycycline for 5 days. The cells were then harvested for protein isolation and immunoblot analysis of p16 expression. As expected, based upon the results of the p16 transient transfections discussed above, the induction of p16 expression in the absence of doxycycline varied widely among the clones. Indeed, of the 29 MCF-7/15-11 clones assessed, 4 exhibited high levels of p16 expression, 3 exhibited moderate levels, 5 exhibited low levels and 17 showed no induction of p16 expression at all. Similarly of the 22 ZR75.1/15-1 clones assessed, 3 exhibited high levels of p16 expression, 4 exhibited moderate levels, 1 exhibited low levels and 14 showed no p16 induction (see

examples in Figure 12). Furthermore, all of the clones that demonstrated moderate to high expression of p16 also underwent a shift from the hyperphosphorylated (inactive) form of pRb which predominated in the presence of doxycycline to a predominantly hypophosphorylated (active) form of pRb in the absence of doxycycline (data not shown). However, in contrast to the complete cessation of growth in cells infected with Ad-p16 adenovirus and expressing extremely high levels of p16, these cells were capable of continued growth. These data indicate that a threshold level of exogenous p16 expression is capable of reducing cyclin D1/CDK4/6 phosphorylation of pRb, and thus contributing to regulation at the G1/S boundary, rather than causing a total inhibition of cell cycle progression as seen in Ad-p16 infected cells. The majority of clones displayed doxycycline-dependence with regard to the degree of p16 expression induced. However, those clones that expressed moderate to high levels of p16 in the absence of doxycycline, also expressed barely detectable to low levels of the p16 protein in the presence of doxycycline. This finding indicates a slight "leakiness" in the Tet-Off system.

Owing to the large number of clones isolated, we have been able to identify three transfectants from each of the two cell lines that express low, moderate and high levels of p16 expression in the absence of doxycycline with zero to barely detectable expression in the presence of doxycycline. We have employed these clones to analyze the effect of different levels of p16 expression upon the *in vitro* and *in vivo* transformed properties of the breast cancer cell lines, MCF-7 and ZR75.1. Among MCF-7 clones we have chosen MCF-7/15-1/p16#29, which exhibits low p16 expression; MCF-7/15-1/p16#69, which exhibits moderate p16 expression; and MCF-7/15-1/p16#13, which exhibits high p16 expression (Figure 12). The latter two clones also express low levels of "leaky" p16 expression in the presence of doxycycline. Among ZR75.1 clones we have chosen ZR75.1/15-1/p16#12, which exhibits low p16 expression; ZR75.1/15-1/p16#7, which exhibits moderate p16 expression; and ZR75.1/15-1/p16#10, which exhibits high p16 expression (Figure 12).

6) Stable transfection, and isolation and analysis of MCF-7/15-1 cells transfected with pTET-SPLICE-antisense cyclin D1

The MCF-7 clone MCF-7/15-1#11, stably transfected with the regulator plasmid pUDH15-1 was also used for stable transfection of the pTET-SPLICE-antisense plasmid. The MCF-7/15-1#11 cells were transfected with pTET-SPLICE antisense cyclin D1 using the protocol described above for stable transfection of pTET-SPLICE-p16. At 4-5 weeks post-transfection, a total of 24 colonies were isolated by ring cloning and transferred to 25 cm^2 flasks. Sixteen of the 24 clones were then assessed for doxycycline-dependent repression of cyclin D1 protein expression. Each of the clones was grown in two 35 mm wells either in the presence or absence of 1 ug/ml doxycycline for 5 days. The cells were then harvested for immunoblot analysis of cyclin D1 expression. Of the 16 clones assessed, 2 showed a reduction in the level of cyclin D1 protein expressed in the absence of doxycycline. Clone MCF-7/15-1/antiD1#2-4 showed a 4-5 fold reduction in cyclin D1 expression, and clone MCF-7/15-1/antiD1#4-2 showed an approximately 3-fold reduction.

Effect of regulated antisense cyclin D1 expression on cell cycle distribution of breast cancer cells

To determine the effect of reduced expression of cyclin D1 protein on cell cycle distribution, we grew the MCF-7 parent cell line and the two clones, MCF-7/15-1/antiD1#2-4 and MCF-7/15-1/antiD1#4-2, in the presence and absence of doxycycline for 7 days. The cells were then harvested and subjected to FACS analysis. One of the clones, MCF-7/15-1/antiD1#2-4, showed an increase in G1 accumulation from 59 to 75 percent and a decrease in S-phase accumulation from 25 to 14 percent in the absence of doxycycline. This indicates that the reduction of cyclin D1 expression in this clone was sufficient to cause a significant but incomplete inhibition of cells entering S-phase. In contrast, clone MCF-7/15-1/antiD1#4-2 did not show a significant difference in cell cycle distribution between cells grown in the presence and absence of doxycycline, suggesting that the 3-fold reduction in the high level of cyclin D1 overexpression that occurs in MCF-7 cells was not sufficient to produce a detectable block in S-phase entry. These data indicate that a reduction in cyclin D1 overexpression is capable of inhibiting the G1 transition, but because of the marginal magnitude of the reduction that occurred, the effects of cyclin D1 ablation on the growth and tumorigenic properties the cells was not analyzed further.

Effect of regulated p16 expression on cell cycle distribution of breast cancer cells

Our analysis of Ad-p16-infected MCF-7 and ZR75.1 cells showed that extremely high levels of p16 protein were capable of causing essentially complete G1 arrest. Our goal, therefore, was to determine the cell cycle distribution in the clones, MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13, following induction of the moderate to high, but not extreme, levels p16 expression seen in these cells. The parent cell line, MCF-7, and each of the three clones were grown in the presence and absence of medium containing 1 ug/ml doxycycline for 7 days, then harvested both for FACS and p16 and pRb expression analysis. Immunoblot analysis indicated the expected degree of p16 induction in the clones grown in the absence of doxycycline (Figure 13). In addition, the moderate and high induction of p16 expression shown by clones MCF-7/15-1/p16#69 and MCF-7/15-1/p16#13, correlated with the presence of the active (hypophosphorylated) species of the pRb protein (data not shown). When these data were compared directly with the cell cycle distribution profiles, it became clear that at least moderate expression of p16 was sufficient to cause a significant accumulation of cells in G1 phase (Figure 13). By contrast, the cell cycle distribution profiles for clone, MCF-7/15-1/p16#29, grown in the presence and absence of doxycycline, were virtually identical. These data confirm that the p16 protein expressed by the MCF-7 transfectants is functional and capable, at a threshold level of inhibiting phosphorylation of pRb protein by cyclin D1/CDK4/CDK6, thereby regulating entry into S-phase. However, as noted above, in contrast to the extremely high levels of p16 expressed in cells infected with Ad-p16, which completely halted the growth of cells, the moderate to high levels produced in cells transfected with Tet p16 constructs were compatible with viability and continued growth. The expression of p16 in Tet p16 transfected cells did, however, suppress the transformed properties of the cells, as shown in the section below.

Effect of regulated p16 expression on transformed properties of breast cancer cell lines

1) Effect of regulated p16 expression on anchorage-independence of breast cancer cell lines

One of the characteristics exclusive to tumor cells is their ability to grow in the absence of anchorage to substrate. It is not known whether p16 plays a role in anchorage dependence as the underlying mechanisms have yet to be fully elucidated. To assay the effect of p16 expression upon the ability of MCF-7 cells to grow in an anchorage-dependent manner, we subjected the clones, MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13, to soft agar analysis. Both the parent cell line, MCF-7, and each of the three transfectants were grown in two 75 cm^2 flasks in the presence and absence of 1 ug/ml doxycycline-containing medium for 7 days, to allow p16 expression to be switched "on" to its full extent in the cultures lacking doxycycline. The cells were then harvested both for p16 expression analysis and soft agar assays. For the analysis of p16 expression, protein extracts were prepared from the same cells as those used for soft agar analysis. All three of the clones showed the expected degree of p16 induction in the absence of doxycycline. For the soft agar assays, MCF-7 cells were suspended in an agar/medium/10% serum mixture plus or minus 1 ug/ml doxycycline, and the transfectants were suspended in an identical mixture supplemented with 500 ug/ml G418 and 500 ug/ml zeocin plus or minus 1 ug/ml doxycycline. A total of 1.25 x 10^4 cells of each cell line were plated onto a layer of agar/medium/10% serum in 8 replicate 60 mm plates. Fresh medium containing the appropriate antibiotic selection plus or minus doxycycline was added to the plates twice per week. After 2.5-3 weeks, the plates were scored for the growth of colonies in soft agar.

The MCF-7 parent cells grew successfully both in the presence and absence of doxycycline forming large, healthy colonies with no evidence of growth suppression in the absence of anchorage. MCF-7/15-1/p16#29 cells also grew to form healthy colonies, though they were fewer and smaller in size than those formed by the MCF-7 parent. Plates containing MCF-7/15-1/p16#29 cells grown in the absence of doxycycline looked similar to those grown in the presence of doxycycline but upon closer scrutiny appeared to contain fewer healthy colonies and more single, inviable cells. The most marked contrast to the MCF-7 parent plates was exhibited by clones MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13. Growth in both the presence and absence of doxycycline resulted in a high frequency of cell death as manifested by the preponderance of inviable single cells and small clusters of 2-8 cells on all of the plates. Essentially no healthy colonies were formed (Table 6). The observation of an insignificant difference between the suppression of growth in soft agar exhibited by cells grown either in the presence or absence of doxycycline suggests that the level of "leaky" p16 expression exhibited by these two clones in the presence of doxycycline was sufficient to inhibit the growth of MCF-7 cells in soft agar. In keeping with this possibility, immunoblots indicate that the Rb protein is predominantly in the hypophosphorylated, (active) state in these cells when grown in soft agar, even in the presence of doxycycline. Even MCF-7/15-1/p16#29 cells grown in the presence of doxycycline, which do not show detectable expression of p16 by immunoblot, appear to be somewhat growth compromised. Two possible explanations are (a) that very low levels of p16 protein expressed in the presence of doxycycline are sufficient to suppress anchorage-independent properties; or (b) that the transfectants themselves are modified in such a way

as to suppress their growth in soft agar, independent of the expression of p16 protein. To address the latter issue, we carried out soft agar analysis on two of the stable MCF-7 Tet clones that were obtained during the course of isolation of p16 expressing clones (MCF-7/15-1/p16#2 and MCF-7/15-1/p16#12), which contain both the pUDH15 regulator and pTET-SPLICE-p16 vectors, but which do not express detectable p16 protein either in the presence or absence of doxycycline. Both of these clones grew in soft agar to form large, healthy colonies that were completely anchorage independent both in the presence and absence of doxycycline (Table 6). These results indicate that the suppression of anchorage independent growth in p16 expressing clones is not due to effects of transfection itself, but appears to directly correlate with the expression (even at very low levels) of p16 protein.

To determine the ability of ZR75.1 cells to grow in an anchorage-independent manner, we subjected three ZR75.1 Tet clones to soft agar analysis. The parent cell line, ZR75.1, and the clones ZR75.1/15-1/p16#12, ZR75.1/15-1/p16#7 and ZR75.1/15-1/p16#10 (which had been found to express low, moderate and high levels of p16 protein in the absence of doxycycline), were grown in two $75cm^2$ flasks in the presence and absence of 1 ug/ml doxycycline-containing medium for 7 days. The cells were then harvested both for p16 expression analysis and soft agar assays. However, in these experiments only clone ZR75.1/15-1/p16#7 showed the expected moderate induction of p16 expression in the absence of doxycycline, with a low level of p16 expression in the presence of doxycycline, as had been observed when these clones were first isolated (Figure 12). In contrast, clones ZR75.1/15-1/p16#12 and ZR75.1/15-1/p16#10 expressed constitutively high levels of p16 protein. The high level of doxycycline-unregulated p16 expression in the latter two clones was consistent with the doxycycline-independent inability of either of the two clones to grow in soft agar (Table 6). The ability of ZR75.1/15-1/p16#7 cells to grow in soft agar did show doxycycline-dependence with a virtually complete failure of those cells cultured in the absence of doxycycline (and expressing a moderate level of p16) to grow in an anchorage-independent manner. This contrasted with the same cells cultured in the presence of doxycycline (and expressing a low level of p16) which, though significantly growth compromised relative to the parent cell line, ZR75.1, were still able to form moderate to large healthy colonies (Table 6). This data supports the p16-mediated suppression of anchorage-independent growth demonstrated by the MCF-7 Tet clones described above, and suggests an important role for p16 inactivation in the development of a transformed phenotype in cells. This experiment also made clear the inherent instability of Tet-regulated expression of p16 in some clones, and highlighted the need to check clones for regulated induction of p16 expression prior to their use in experimental assays.

2) Effect of regulated p16 expression on the tumorigenic properties of MCF-7 breast cancer cells

The clones MCF-7/15-1/p16#69 and MCF-7/15-1/p16#13, whose ability to grow in soft agar was suppressed by the expression of p16, were chosen to investigate the ability of p16 to suppress the growth of MCF-7 breast tumors in nude mice. Initially, we assayed the tumorigenicity of the MCF-7 parent cell line. As noted above, the formation of tumors in

nude mice by MCF-7 cells is estrogen dependent. We therefore implanted one 1.7 mg pellet of 17 B-Estradiol (Innovative Research of America) into each of three nude mice, subcutaneously between the right shoulder and head. The pellet releases a constant daily level of 17 B-Estradiol to produce a blood concentration of 500-600 pg/ml, reaching a steady state concentration approximately 48 hours post-implantation. The mice were then injected with 10^7 MCF-7 cells, subcutaneously into the left shoulder. Eight 175 cm^2 flasks were grown of each of the MCF-7 clones MCF-7/15-1/p16#69 and MCF-7/15-1/p16#13, expressing moderate and high levels of p16 in the absence of doxycycline, respectively (Figure 12), and doxycycline removed from the medium 7 days prior to injection, to induce p16 expression. The flasks were then harvested and the cells used both to inject each of 3 mice subcutaneously behind the shoulder with 10^7 cells per mouse, and to prepare protein extracts. Both of the clones showed appropriate induction of p16 protein expression relative to control cells cultured in the presence of doxycycline. Furthermore, unlike the MCF-7 cells that formed tumors of 0.3 to 0.45 cm diameter within 8 weeks, neither of the two p16-expressing clones had developed detectable tumors by 12 weeks post-injection (Table 7). This indicated that the expression of moderate to high levels of p16 protein was sufficient to completely inhibit tumor growth in nude mice, thus confirming the results of the *in vitro* soft agar studies. Thus p16 expression in MCF-7 cells, at a level compatible with viability and growth of cells under normal culture conditions, reversed the transformed phenotype of these cells, suppressing both growth in soft agar and tumorigenicity in nude mice.

RESULTS - SECTION III

FURTHER CHARACTERIZATION OF CELL CYCLE REGULATORY PROTEIN EXPRESSION IN BREAST TUMOR TISSUES

Status of the p16 gene in breast tumor tissues

Section I of this report presented the analysis of a panel of 10 pairs of matched normal and tumor breast tissues for the expression of the G1 phase regulatory proteins, cyclin D1, Rb and p16. Like the breast cancer cell lines previously analyzed, all 10 tumors demonstrated a correlation between cyclin D1 and Rb expression. Specifically, 8 of the 10 tumor tissue extracts expressed pRb and overexpressed cyclin D1, while two of the tumors failed to express both Rb and cyclin D1 (Table 4). In addition, we were unable to detect the expression of p16 in any of these tumors. Thus the tumors appeared to have the same type of defects in cell cycle regulation that we had identified in breast cancer cell lines. The apparent lack of p16 protein expression in the tumor tissues suggested the possible inactivation of the p16 gene, perhaps by homozygous deletion or methylation. We therefore subjected DNA isolated from microdissected cells corresponding to the 10 pairs of normal and tumor cells, to methylation-specific PCR (MSP) analysis at the p16 locus. This type of analysis reveals whether a particular gene is present in the genome, and if present, whether it exists in a methylated or unmethylated state. Normal and tumor DNA was modified overnight using sodium bisulfite to convert all of the unmethylated cytosines to uracil. The resulting DNA was then subjected to PCR analysis using oligonucleotides designed from the promoter of the p16 gene that were specific for wild type (unmodified), methylated and unmethylated DNA (Herman et al., 1996). As expected, all of the normal samples demonstrated amplification using the unmethylated-specific primers only. Surprisingly, however, all 10 of the tumor samples also underwent amplification with the unmethylated primers (representative data in Figure 14). This result indicated that none of the tumors were homozygously deleted for the p16 gene since all were successfully amplified, and that the p16 gene was not transcriptionally silenced in these tumors due to promoter methylation. The lack of p16 gene inactivation by deletion or methylation leaves open the possibilities that (a) the p16 gene is inactivated by another mechanism including deletion of one allele and mutational inactivation of the remaining allele (loss of heterozygosity); or (b) p16 is expressed in breast tissues at a level that is not detectable by the immunoblot procedure we employed. The fact that p16 was also not detected in normal breast tissue by our immunoblotting procedure suggested that the second possibility might be correct. To address this possibility, we explored the use of a newly available p16 antibody in the immunoblot procedure, to determine whether it might be sensitive enough to detect p16 in normal and breast tumor tissues.

Expression of p16 protein in breast tumor tissue

Using a new, more sensitive p16 antibody (Oncogene) in addition to previously used antibodies to Rb and cyclin D1, we have subjected a larger breast tissue panel to immunoblot analysis for these proteins. The panel consists of 7 of the previously described 10 pairs of normal and tumor tissues (Table 4), plus an additional 5 new pairs of matched normal and tumor tissues. Owing to the lack of tissue availability, the other three pairs of tissues previously studied could not be subjected to further analysis. With this more sensitive antibody, p16 was found to be absent or present at only barely detectable levels in 2 of the 12 breast tumor tissues (Table 8). These 2 tumors also expressed cyclin D1 and Rb, and therefore appeared to have a defect in cell cycle regulatory similar to that found in the majority of breast cancer cell lines, namely expression or overexpression of cyclin D1 in the presence of Rb and the absence of p16. The remaining 10 of the 12 tumor tissues were found to express readily detectable levels of p16 protein (Table 8, representative immunoblots are shown in Figures 14 and 15). In one case the expression of p16 was accompanied by a failure to express Rb, indicating a defect in cell cycle regulation comparable to that found in 3 of the breast cancer cell lines studied (Table 1). In the absence of Rb, the expression of p16 and the absence of cyclin D1 seen in this tumor would not be relevant to loss of cell cycle control.

However, in 9 of the tumors expressing p16, Rb was also expressed, accompanied by the expression or most frequently the overexpression of cyclin D1 (Table 8). Therefore the pattern of expression seen in these tumor tissues does not correspond to the types of defects we observed in breast cancer cell lines. Overexpression of cyclin D1 does occur in most of these tumors, but is frequently accompanied by overexpression of p16 protein, relative to the corresponding matched normal tissue, as well (Table 8). Overall the data do not appear to indicate defects in cell cycle regulatory components that would clearly lead to loss of G1/S boundary regulation in the majority of the breast tumors studied. However, further analysis of the cell cycle regulatory defects which occur in breast cancer cell lines, as described in the next section, suggests a mechanism which might contribute to loss of cell cycle control in those tumors expressing p16 together with Rb and cyclin D1.

RESULTS - SECTION IV

IDENTIFICATION OF AN UNUSUAL DEFECT IN CELL CYCLE REGULATION IN BREAST CANCER CELL LINES

Detection of a subset of breast cancer cell lines that coexpress Rb and p16

The results described in the previous section of this report indicate that the majority of breast tumors we examined do not have the types of defects in cell cycle regulation that we found most frequently in breast cancer cell lines. Most of the tumors were in fact similar in their expression of cell cycle regulatory proteins to the exceptional cell line MDA-MB-157. This cell line appeared to be the single exception to the patterns of defects seen in other cells, in that it expressed cyclin D1 in the presence of Rb plus p16, and thus did not appear to have a defect in cell cycle regulatory protein expression. However, because the more sensitive p16 antibody had revealed the presence of p16 in tumor tissues in which it had previously been undetectable, we reexamined our panel of breast cancer cell lines using this antibody. In addition, we added two new cell lines, COLO 742 and MDA-MB-134IV, to our panel for a total of 14 cell lines. Immunoblotting for p16 with this more sensitive antibody revealed that cell line MDA-MB-415, previously scored as p16 negative, in fact contained readily detectable p16. COLO 742, like MDA-MB-157 and MB-MBA-415, also expressed cyclin D1 in the presence of Rb plus p16, giving a total of three cell lines that appeared similar to the pattern of expression seen in most breast tumor tissues (Figure 16 and Table 9). The second new cell line, MDA-MB-134IV, overexpressed cyclin D1 in the presence of Rb and failed to express p16 (Figure 16), thus showing the same pattern of defects seen most frequently in the breast cancer cell lines studied earlier.

Identification of breast cancer cell lines insensitive to cell cycle block by p16 inhibitor protein

The co-expression of cyclin D1, Rb and p16 observed in most breast tumor tissues and in 3 of 14 breast cancer cell lines examined suggests the existence of alternate mechanisms for overcoming cell cycle regulatory control in breast cancer cells. One possibility is the presence of defects in the p16 protein expressed in these cells that would render it inactive as an inhibitor of cell cycle progression. A second possibility is the presence of defects that would cause the cells to be insensitive to the inhibitory actions of fully functional p16. We had previously shown that p16 expressed from the Ad-p16 adenovirus construct was capable of completely blocking MCF-7 and ZR-75.1 cells in G1 and halting growth (see Figure 9), demonstrating that the failure to express p16 in these cells contributed to the loss of cell cycle control. To test whether breast cancer cell lines expressing p16 in the presence of Rb and cyclin D1 are sensitive to the expression of known functional p16, we infected MDA-MB-157 and COLO 742 cells with the Ad-p16 adenovirus construct. As controls, we also infected HBL-100 cells, which contain SV-40 T-antigen

inactivated Rb and thus would be expected to be insensitive to p16, and MCF-7 cells, which are blocked by p16 as shown previously. The cells were also infected with an adenoviral construct expressing green fluorescent protein (Ad-GFP), to indicate both the infection efficiency of the virus and the effects of adenoviral infection alone on the growth characteristics of the cells. Forty hours after infection, the uninfected and infected cells were analyzed both for cell cycle distribution by FACS, and the expression of p16 and several other cell cycle regulatory proteins by immunoblot analysis. Following infection with Ad-GFP all cell lines expressed extremely high levels of GFP protein indicating a high (>95%) infection efficiency (data not shown). In addition, the Ad-GFP-infected cells showed exactly the same cell cycle distribution profile as the uninfected logarithmic phase cultures indicating that the adenoviral construct lacking the p16 gene had no effect upon the growth characteristics of the cells. As shown in Figure 17, p16 was expressed at very high levels in all infected cell lines. As expected, MCF-7 cells were completely blocked in G1. In contrast, the growth and cell cycle distribution of MDA-MB-157 and COLO 742 cells were not significantly affected by p16 expression, just as was the case for the control p16-insensitive HBL-100 cells (Figure 17). The cell cycle distribution profile for Ad-p16 infected MDA-MB-415 cells, the third breast cancer cell line that co-expressed cyclin D1, Rb and p16, was intermediate between that of the p16-sensitive MCF-7 and the MDA-MB-157 and COLO 742 p16-insensitive cells (data not shown). MDA-MB-415 cells express a somewhat lower level of p16 protein than the highly p16-insensitive MDA-MB-157 and COLO 742 cells, suggesting that these cells may be only partially insensitive to p16.

Immunoblot analysis of cell cycle regulatory protein expression supported the cell cycle distribution data. As shown previously, MCF-7 (Figure 9) and ZR75.1 cells infected with Ad-p16 showed a significant decrease in cyclin D1 expression, consistent with the binding of p16 to CDK4 and CDK6, and subsequent degradation of monomeric cyclin D1 protein. This correlated with a shift in the expression of Rb protein to the fully active hypophosphorylated state, and arrest in G1 in the Ad-p16-infected cells. The inability of MCF-7 cells to enter S-phase was also illustrated by the exceptionally low levels of cyclin A, a protein which is ordinarily expressed upon entry into S-phase and maintained throughout G2/M, and the complete absence of the mitotic cyclin, B1 (Figure 9). These data indicate that the loss of p16 expression is the rate-limiting defect of the Rb/cyclin D1/p16 pathway in the MCF-7 and ZR75.1 cell lines, since Rb-mediated G1 arrest can be restored by overexpression of ectopic functional p16 protein.

In contrast, we observed no significant changes in the expression of any the above cell cycle regulatory proteins in the HBL-100, COLO 742 and MDA-MB-157 cell lines, consistent with their unperturbed cellular proliferation. Representative immunoblot data for MDA-MB-157 is shown in Figure 18. We also did not observe a significant change in the ratio of hyperphosphorylated to hypophosphorylated Rb protein species in the uninfected MDA-MB-157 cells versus the Ad-p16-infected cells (Figure 18). This indicates that the ectopic p16 protein was unable to affect the activity of cyclin D1/CDK4-6 holoenzymes in these cells, possibly due to mutations of CDK4 or CDK6 that inhibited p16 binding. Ectopic overexpression of functional p16 protein in HBL-100 cells resulted in a shift of Rb protein to the fully hypophosphorylated state indicating the successful binding of p16 to CDK4-6 and inhibition of cyclin D1/CDK4-6 activity (data not shown). However, because the HBL-100

cell line is transformed with SV40, the Rb protein is inactivated and therefore unable to bind to E2F and establish a G1 arrest.

The findings presented above indicate that a subset of breast cancer cell lines escape cell cycle regulatory control by some mechanism that renders them insensitive to inhibition by p16. The fact that the majority of breast tumor tissues we examined co-express cyclin D1, Rb and p16, as do the p16-insensitive cell lines MDA-MB-157, COLO742 and MDA-MB-415 suggests the possibility that a similar defect in cell cycle regulation may occur in breast tumors. The high frequency of cyclin D1, Rb and p16 co-expression seen in our breast tumor samples also suggests that this type of defect may occur more frequently in primary breast tumors than the types of defects involving loss of p16 or Rb identified in most of the breast cancer cell lines. In addition, as discussed below, our studies of cell cycle regulatory defects in ovarian tumor tissue and cell lines indicate that this type of defect may not be confined to breast cancer cells.

DISCUSSION AND CONCLUSIONS

Section I

The results presented in Section I of this report demonstrate that defects of varying degrees and types in the expression of cell cycle regulatory proteins occur with one exception in all breast cancer cell lines and tumors examined. In cell lines, the most common defect observed was the expression or overexpression of cyclin D1 in the presence of Rb, accompanied by failure to express p16 (Table 1). The fact that some breast cancer cell lines expressed normal levels of cyclin D1 suggests that the loss of p16 is the fundamental defect contributing to loss of cell cycle regulatory control in these cells. This conclusion is supported by observations of the effects of p16 expression constructs on the transformed properties of breast cancer cell lines overexpressing cyclin D1 (see Section II). The one exceptional cell line identified in this phase of the study, expressing normal levels of cyclin D1, Rb and p16, was later shown to contain an unusual defect in cell cycle regulatory components (see Section IV). The remainder of the cell lines studied failed to express Rb, thereby rendering the expression or lack of expression of cyclin D1 and p16 irrelevant to the loss of cell cycle control. These findings parallel earlier studies in our laboratories which showed that the same two kinds of defects occur in different types of lung cancer cell lines (Schauer et al., 1994), and which provided an early indication that loss of cell cycle regulation at the G1/S boundary by one mechanisms or another may be a necessary step in progression of cells to the malignant state.

In breast tumor tissues we also observed that overexpression of cyclin D1 in the presence of Rb was the most common defect, with only a few tumors in our panel showing loss of Rb (Table 4). In the absence of an ability to detect p16 protein in breast tumor tissue in these early studies, we initially interpreted these results to indicate that overexpression of cyclin D1 in the presence of Rb, accompanied by failure to express p16 was the most common mechanism for loss of cell cycle regulation in both breast tumors and breast cancer cell lines. However, reexamination of cell cycle regulatory protein expression in breast tumors with improved technology has led to a revision of this conclusion (see Sections III and IV). While this work was being planned and carried out, a number of other laboratories carried out studies of the expression of cyclin D1, Rb and p16 in breast cancer cell lines (Bartkova et al., 1994; Tam et al., 1994) and tumors (Gillet et al., 1994; Zhang et al., 1994; Bartkova et al., 1994; Bartkova et al., 1995; Zukerberg et al., 1995; Geradts et al., 1995, 1996). However, in none of these investigations were all three proteins involved in the cyclin D1-Rb-p16 regulatory pathway examined simultaneously in the same cell lines or tumor samples, as was done in our studies. Nevertheless, from this work as well as work in many laboratories on cell cycle regulatory protein expression in other types of cancer, it emerged as is now well recognized that essentially all cancer cells have defects of some kind in regulation of cell cycle progression at the G1/S boundary by the cyclin D1-Rb-p16 system (Bartek et al., 1997).

Our studies on the mechanisms responsible for overexpression of cyclin D1 have shown that overexpression is associated with a combination of cyclin D1 DNA amplification

and mRNA overexpression, but that in some cases these mechanisms do not explain the degree of cyclin D1 overexpression (Table 2), indicating that other mechanisms contribute. In one breast cancer cell line, an increase in the half-life of cyclin D1 was shown to account for the increased cyclin D1 expression observed (Figure 2), indicating that changes in the regulation of cyclin D1 protein degradation contribute to overexpression in this case. A change in the degradation rate of cyclin D1 in association with overexpression has been reported in only one other case, that of uterine sarcoma cell lines (Weleker et al, 1996). Expression of cyclin D1 in breast cancer cell lines was also found to be independent of regulation by growth factors (serum) or cell-cell contact (Figures 3 and 4), which may also contribute to overexpression of this protein.

Failure to express p16 in our panel of breast cancer cell lines is associated with homozygous deletion or methylation of the p16 gene in 70 percent of cases (Figures 5 and 6). These mechanisms have been shown to be responsible for lack of p16 expression in a wide variety of cancers, as noted in Section I of Results. The mechanism causing failure of p16 expression in the remaining cell lines has not been determined, but may involve mutation followed by loss of heterozygosity.

Section II

In Section II we have described our studies aimed at direct demonstration of the contribution of defects in cyclin D1 and p16 expression to the malignant characteristics of breast cancer cell lines. Using constructs in which the expression of p16 and of antisense cyclin D1 is under control of a tetracycline-regulated transcriptional activator, we have shown that antisense cyclin D1 lowers cyclin D1 expression and marginally induces an increase in G1/S blockade in a breast cancer cell line overexpressing cyclin D1. The marginal effect on cell cycle regulation in cells expressing antisense cyclin D1 may again be an indication that the fundamental defect in these cells leading to loss of cell cycle regulatory control is the failure to express p16 rather than the overexpression of cyclin D1.

Transfection of p16 expressing constructs into breast cancer cell lines not expressing this protein has been shown to produce moderate levels of p16 expression and greatly increase G1/S blockade in these cells (Figure 13), as well as to suppress anchorage-independent growth (Table 6) and tumor formation in nude mice (Table 7), without substantially affecting growth or viability of the cells under normal culture conditions. Therefore restoration of p16 expression in breast cancer cells reverses two important feature of the transformed phenotype in these cells, directly demonstrating the contribution of p16 protein deletion to the malignant state of the cells. With respect to the relative importance of cyclin D1 overexpression and p16 deletion in tumorigenesis, it is important to note that suppression of transformed properties is brought about by the expression of low to moderate amounts of p16 in cells that strongly overexpress cyclin D1 (10-fold), indicating that overexpression of cyclin D1 by itself does not confer loss of cell cycle control and transformed properties on cells.

Section III

Reexamination of an expanded panel of breast tumor tissues for expression of cell cycle regulatory proteins (Table 8) showed a low proportion of Rb protein deletion and a high proportion of cyclin D1 overexpression, as observed previously by us (Table 4) and others (T'Ang et al., 1988; Varley et al., 1989; Bartkova et al., 1994; Geradts et al., 1995, 1996). However, the surprising findings that emerged from this reexamination were the existence of the p16 gene in all the tumors in an undeleted and unmethylated state (Figure 14) and the expression or overexpression of p16 protein in 9 of the 11 tumors expressing Rb (Figures 14 and 15; Table 8). Thus, these tumor tissues differed strikingly in their expression of cell cycle regulatory proteins from all but one of the breast cancer cell lines examined (Table 1). The coexpression of Rb and p16 has rarely been observed in other types of tumor tissues. However, in breast cancer tissues Geradts et al. (1995, 1996) have previously reported a significant proportion of breast tumors coexpressing Rb and p16. Although in these reports the investigators placed emphasis on the proportion of tumors that failed to express p16, and did not specifically comment on the unusual occurrence of tumors coexpressing p16 and Rb, it is clear from the data presented that at least 38 of 104 breast tumors examined express both Rb and p16. In our own breast tumor tissue samples, the proportion coexpressing Rb and p16 was higher (9 of 12 total), but this discrepancy is not large considering the relatively small number of tumors we examined.

Expression of p16 mRNA has also been reported to occur in greater than half the breast tumor tissues studied by Brenner et al. (1996), although again these authors placed emphasis on those tumors not expressing p16. The status of Rb expression was not examined in this study, but given the well-documented low frequency of Rb inactivation in breast cancers, it seems highly likely that many of the breast tumors examined in this study also coexpressed both Rb and p16. Finally, Van Zee et al. (1998) also have found that the p16 gene is present in an undeleted and unmethylated state in a high proportion of breast tumor tissues, and that this is accompanied by expression of p16 mRNA in these tumors. On the basis of their findings, these investigators have questioned the importance of the failure to express p16 in the development of breast cancer.

Since, as noted above, our studies of breast cancer cell lines have strongly indicated that, in cells expressing Rb, it is the loss of p16 rather than the overexpression of cyclin D1 that is crucial for the loss of cell cycle regulation and the development of transformed properties, the finding of coexpression of Rb and p16 in breast tumor tissues raises the question as to whether defects in cell cycle regulation are in fact present in a substantial proportion of breast tumors. However, the findings reported in Section IV reveal that a subset of breast cancer cell lines also coexpress Rb and p16, but escape cell cycle regulation by a mechanism other than the loss of one of these proteins. This suggests the possibility that an unusual mechanism may be involved in the loss of cell cycle control in a substantial proportion of breast tumors.

Section IV

Reexamination of an expanded panel of breast cancer cell lines for the expression of cell cycle regulatory proteins revealed that a subset of these cell lines (3 of 14) also coexpressed Rb and p16 (Table 9), as did the majority of breast tumor tissues examined. In order to determine whether defects in cell cycle regulation occurred in these cells in spite of the expression of a near normal complement of cyclin D1, Rb and p16, we used the adenoviral p16 construct Ad-p16 to test the response of these cells to the expression of very high levels of p16. p16 expressed by Ad-p16 was known to be functional, due to its ability to block breast cancer cell lines not expressing p16 in G1 (Figure 9) and stop their growth. On expression of very high levels of p16 in those breast cancer cell lines that expressed endogenous p16 in the presence of Rb, two of the cell lines were found to be completely insensitive (Figures 17 and 18) and one of them partially insensitive to blockade of cell cycle progression by p16. This indicates a defect in cell cycle regulation in these cells that is downstream of p16 and which, importantly, is not revealed by an analysis of the levels of cyclin D1, Rb and p16 in these cells. Since we observed that expression of very high levels of p16 in these cells did not significantly change the proportion of Rb in the hyperphosphorylated state, this suggest that these cells might contain an altered form of CDK4 or CDK6 which is insensitive to inhibition by p16. A CDK4 mutation that results in a cys-arg transition in CDK4 which renders it unable to bind and be inhibited by p16 has been reported in melanoma tumors (Wolfel et al., 1995; Zuo et al., 1996). However, we have examined a p16 insensitive cell line for this CDK4 mutation and found it not to be present (M. C. Todd, unpublished data). However other CDK4 mutations or CDK6 mutations could contribute to p16 insensitivity in this cell line. Other mutations downstream of p16 could of course cause insensitivity to cell cycle block by this inhibitor, but the fact noted above that high levels of exogenous p16 do not affect the phosphorylation state of Rb strongly points to some type of p16-insensitive kinase activity. In any case, the demonstration of p16 insensitivity in breast cancer cell lines, coupled with the frequent coexpression of Rb and p16 observed in breast tumors by us and others suggests the possibility that defects in cell cycle regulation of this kind may make a substantial contribution to breast cancer tumorigenesis. That the existence of this type of defect has gone unrecognized may be attributed to the fact that simple examination of the levels of cell cycle regulatory proteins in these tumors reveals little deviation from normal tissue, other than a varying degree of cyclin D1 overexpression. Only the study of p16 insensitivity in breast cancer cell lines coexpressing Rb and p16 made the presence of a defect evident, and raised the possibility that the same defect might occur in breast tumors. In addition, in a parallel study of cell cycle regulatory protein expression in ovarian tumors, we have found that approximately half of these tumors also coexpress Rb and p16, and that several ovarian cancer cell lines that coexpress these proteins are insensitive to the expression of high levels of p16 by the adenoviral Ad-p16 construct (M. C. Todd and T. A. Langan, manuscript in preparation). Thus, loss of cell cycle regulatory control in cancer cells by mechanisms other than the deletion of p16 or Rb may be more widespread than has been appreciated, and may make an important contribution to oncogenesis in several specific types of cancer.

Summary of implications for current understanding and future work

During the time since this work was first proposed, the widespread occurrence of defects in cell cycle regulation in malignant cells has been extensively documented by studies in a large number of laboratories. Our contributions to this general documentation have therefore been relatively minor, partly due to the fact that key findings were made by others before and shortly after initiation of our funding. However, by carrying out a more comprehensive investigation of cell cycle regulatory than has been done in most investigations, in particular with respect to examining of all three of the key components, cyclin D1, Rb and p16 in the same study, we have added to the understanding of the importance of different types of cell cycle regulatory defects. First of all, in breast cancer cell lines, which in spite of their disadvantages must be continued to be used for investigations of tumorigenesis, the loss of p16 rather than the overexpression of cyclin D1 appears to be the most important defect. This is indicated by the fact that in breast cancer cells expressing Rb, expression of p16 suppresses the transformed phenotype of the cells, while reduction in cyclin D1 levels to near normal levels has only a marginal effect on cell cycle regulation and growth properties. Second, in spite of the importance of p16 loss for transformed properties of breast cancer cell lines, our comparison of cyclin D1, Rb and p16 expression in breast tumor tissues has indicated that loss of p16 is of considerably less importance in the large majority of breast tumors expressing Rb, and emphasized the unrecognized importance of observations made by others of the coexpression of Rb and p16 in breast tumors. Our investigation of breast cancer cell lines having the same near-normal pattern of cell cycle regulatory protein expression found in many breast tumors has revealed an unusual defect in cell cycle regulation in these cells, namely insensitivity to the inhibitory effects of p16. It is now essential in future work to unequivocally identify the basis for this insensitivity, and to determine whether this or other defects are present in the large proportion of breast tumors which express p16 in the presence of Rb. In addition, other studies ongoing in our laboratory indicate that similar unusual defects may be frequent in ovarian cancer, and that future work should be directed towards characterizing the importance and frequency of occurence of such defects in other types of cancer.

RELATIONSHIP TO STATEMENT OF WORK

During the third year of funding, we submitted a revised SOW, in order to accommodate developments in the area of research covered by the grant. The new SOW included a significant expansion of the original objectives, in particular with respect to the investigation of the newly discovered CDK inhibitor protein p16.

The work carried out since the submission of the revised SOW has expanded still further, again to accommodate new developments made by us and others. In the description given below, bold face indicates work carried out over and above the original SOW, and underlining indicates work in addition to that specified in the revised SOW. One task specified in the original SOW, that of determining the effects of antisense Rb expression, has been eliminated. As described in connection with the submission of the revised SOW, research developments had made the result of such experiments a foregone conclusion.

Task 1. Characterize breast cancer cell lines with respect to overexpression of cyclin D1 protein and the presence of Rb (retinoblastoma) protein, as well as cyclins A, B and E and cyclin-dependent kinases 2 and 4, using immunoblotting techniques. **In addition, characterize these cell lines with respect to the expression of p16 inhibitor protein, as well as inhibitors p21, p27 and cyclin-dependent kinase 6.**

This work has been carried out as described in Sections I and IV of Results.

Task 2. Determine whether cyclin D1 overexpression in breast cancer cell lines is independent of normal growth factor/serum requirements, and therefore not regulated by normal growth control mechanisms. **In addition, investigate whether cyclin D1 expression is affected by cell-cell contact as determined by growth of breast cancer cells to confluence.**

This work has been carried out as described in Section I.

Task 3. Stably transfect breast cancer cell lines overexpressing cyclin D1 with plasmids expressing antisense cyclin D1 constructs and determine their effect on cyclin D1 levels and on the proliferative and transformed properties of the cells. **In addition, stably express p16 sense constructs in breast cancer cell lines not expressing p16, and determine effects on proliferative and transformed properties.**

This work has been carried out as described in Section II. Because the effects of antisense cyclin D1 on cell proliferative properties were found to be marginal, its effects on transformed properties of breast cancer cell lines were not investigated.

Task 4. Examine surgically obtained breast cancer tissue for cyclin D1 protein expression and Rb protein content. **In addition, examine breast cancer tissue for the expression of p16 protein.**

This has been carried out as described in Sections I and III.

Task 5. Characterize the complexes formed by cyclin D1 with cyclin-dependent kinases in normal and breast cancer cell lines.

This has been carried out as described in Section I. As noted in Section I, because of technical difficulties, the extent of this analysis was more limited than originally proposed.

Task 6. Determine whether the overexpression of cyclin D1 in breast cancer cells results from gene amplification, from increased mRNA synthesis, or from a decrease in the rate of degradation of cyclin D1 protein.

This has been carried out as described in Section I.

Task 7. Determine whether the lack of p16 expression in breast cancer cells and tumor tissues is due to homozygous deletion or methylation of the p16 gene.

This has been carried out as described in Sections I and III.

Task 8. Determine whether breast cancer cell lines coexpressing Rb and p16 are sensitive or insensitive to the inhibitory actions of ectopically expressed, functional p16 protein.

This has been carried out as described in Section IV.

Materials and Methods

Breast cancer cell lines and tumor material

A panel of 14 breast cancer cell lines were obtained both from the American Type Culture Collection (DU4475, MDA-MB-134VI, MDA-MB-157, MDA-MB-175VII, MDA-MB-361, MDA-MB-415 and Hs578T), and the University of Colorado Tissue Culture Core Facility (COLO 591, COLO 742, MCF-7, MDA-MB-231, MDA-MB-330, T-47D and ZR75.1). One normal, immortalized, non-transformed breast epithelial cell line, MCF-12A, and one normal breast epithelium cell line transformed with SV40 T antigen (TAg), HBL-100, were also obtained from the University of Colorado Tissue Culture Core Facility. The cell lines COLO 591, COLO 742 and DU4475 were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 18 mM HEPES, 1% non-essential amino acids, 1 mM sodium pyruvate and 2 mM L-glutamine. MCF-12A was cultured in Ham's F12/DME (1:1) supplemented with 10% fetal calf serum, 20 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and 10 ug/ml insulin. Hs578T was cultured in DMEM media supplemented with 10% fetal calf serum, 0.45% glucose, 0.4% sodium bicarbonate and 2 mM L-glutamine. The remaining cell lines were cultured in MEM media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 10 mM HEPES, 1% non-essential amino acids, 2 mM L-glutamine and 6 ng/ml insulin. Fifteen matched pairs of frozen normal and tumor tissues were obtained from breast cancer patients by Dr. Wilbur Franklin, Dept. of Pathology, University of Colorado Medical School.

Antibodies

The following primary antibodies were obtained from Upstate Biotechnology, Lake Placid, NY: anti-cyclin A, anti-cyclin B1, anti-cyclin D1, anti-cdk2, anti-cdk4, anti-cdk6, and anti-PSTAIRE kinase. Anti-cyclin E was obtained from Pharmingen. Two p16 antibodies were employed in our studies. The first was obtained from Pharmingen and the second from Oncogene. Anti-pRb and PCNA were gifts from Dr. Wen-Hua Lee (University of Texas Health Science Center, San Antonio, TX) and Dr. Wilbur Franklin (University of Colorado Health Sciences Center, Denver, CO) respectively. Anti-p21 and anti-p27 were obtained from Santa Cruz. The horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad.

DNA probes and oligonucleotides

A 1.1 kb cDNA probe, pPl-8, was obtained from Invitrogen. The B-actin probe was a gift from Dr. Robert Gemmill (University of Colorado Health Sciences Center, Denver, CO).The c5.1 (p16) oligonucleotide sequences were obtained from Kamb et al., (1994) and those corresponding to the D9S199 locus were obtained from the Genome Data Base. The pUDH15-1 oligonucleotide primer sequences are as follows:

Sense primer: 5' TAG ATG TGC TTT ACT AAG TC 3'

Antisense primer: 5' ACT TGA TGC TCT TGA TCT TC 3'

The p16 exon 2 oligonucleotide primer sequences are from Hussussian et al., 1994.

Adenoviral expression constructs

The Ad-p16 viral construct was obtained from Introgen, and the Ad-GFP viral construct was a gift from Dr. Jerome Schaack (Department of Microbiology, University of Colorado Health Sciences Center).

Tet-expression system plasmid constructs

The Tet "regulator" (pUDH15-1) and "response" (pTET-SPLICE) plasmids, were a gift from Dr. Bujard (Heidelberg, Germany).

Protein extraction and western blot analysis

Cells were harvested from each of two 175 cm^2 flasks at 50-70% confluence. Following two washes in PBS, approximately 2 X 10^6 cells were removed for fluorescence-activated cell sorter (FACS) analysis by the University of Colorado Cancer Center Flow Cytometry Core to determine cell cycle distribution. The remaining cells were resuspended in Laemmli sample buffer (Laemmli., 1970), boiled for 4 minutes, sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C. Individual pieces of tumor and normal tissues each weighing approximately 0.2 g, were crushed to a fine powder under liquid nitrogen, lysed in Laemmli sample buffer and processed as described above for the cell lines.

Approximately 50-100 ug of each protein extract were subjected to SDS/PAGE (Laemmli., 1970) and transferred either to nitrocellulose (Schleicher and Schuell) or Immobilon P (Amersham) membranes for 45 minutes at 0.45 A using the Genie Electrophoretic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading, and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Fluorescent activated cell sorter (FACS) analysis

Cells were harvested and washed twice in PBS. A minimum of 3 X 10^3 cells were then resuspended in 0.5 ml Krishan's stain (Krishan, 1975) and subjected to cell cycle distribution analysis by the FACS core of the Cancer Center, University of Colorado Health Sciences Center.

Purification of GST-Rb-(C) substrate

A 10 ml culture of E.coli, transformed with the plasmid expressing the GST-Rb-(C) fusion protein, was grown to saturation overnight. The cells were then used to seed a 500 ml culture which was grown for 2 hours at 30°C. Expression of the GST-Rb-(C) protein was induced by the addition of 1 mM IPTG and incubation at 30°C overnight. The cells were then harvested and lysed on ice by sonication in STE (50 mM Tris pH 7.5, 120 mM NaCl, and 1 mM EDTA) containing 1 mM DTT, 0.1 mM PMSF, 5 ug/ml leupeptin, and pepstatin. Precleared lysates were then mixed with 50% glutathione-sepharose overnight at 4°C. The beads were washed once with 10 ml STE/1% triton containing 1 mM DTT and 100 mM PMSF, once with 10 ml STE containing 1 mM DTT and 100 mM PMSF, twice with 10 ml STE containing 0.5 mM DTT and 50 mM PMSF, and twice with 10 ml 50 mM HEPES containing 0.5 mM DTT and 50 mM PMSF. The GST-Rb-(C) protein was eluted in four stages using 0.5 ml kinase buffer (50 mM HEPES

pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA) containing, 10 mM B-glycerophosphate, 0.1 mM sodium orthovanadate) containing 2 mM reduced glutathione. Ten microliter aliquots of each of the four elution products were subjected to electrophoresis on a 10% polyacylamide gel with protein standards of known concentration to assess both the substrate purity and concentration.

Immune complex kinase assays

Logarithmic phase cells were harvested from one 10 cm plate with 1 ml of HEPES/Tween lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM DTT, 0.1% Tween-20) containing 10 mM B-glycerophosphate, 0.1 mM sodium orthovanadate, 2 ug/ml aprotinin, 5 ug/ml leupeptin and 0.1 mM PMSF, and snap frozen in liquid nitrogen. Following thawing on ice, the lysates were clarified by centrifugation at 15 k rpm for 10 minutes at 4°C. Aliquots of the lysates were quantitated using the BCA protein assay kit (Pierce) and the remaining lysates precleared by mixing each milligram of protein with 20 ul of Protein A beads for 30 minutes at 4°C. Immunoprecipitation reactions were then performed by mixing 250 ug of each lysate with 20 ul of a 50/50 slurry of antibody precoated Protein A beads, and incubation at for 2 hours at 4°C. As a negative control, each lysate was also immunoprecipitated with Protein A beads precoated with normal rabbit serum. The immunoprecipitated protein on the beads was then washed four times with 1 ml of cold HEPES/Tween lysis buffer, and two times with 1 ml of cold 50 mM HEPES pH 7.5/1 mM DTT. The beads were then resuspended in 30 ul of kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA) containing, 10 mM B-glycerophosphate, 0.1 mM sodium orthovanadate, 20 uM cold ATP, 10 uCi gamma ^{32}P(ATP) and 1 ug GST-Rb-(C). The reactions were incubated at 30°C for 30 minutes with occasional mixing, boiled in 30 ul 2 x SDS-PAGE loading buffer, and fractionated on a 10% SDS-polyacrylamide gel. After Coomassie staining, the gel was dried down and exposed for autoradiography.

DNA isolation and Southern blot analysis

DNA was prepared by incubating cells or finely minced solid tissues at 55°C in lysis buffer (10 mM Tris pH 8.0, 2.0 mM EDTA pH 8.0, 10 mM NaCl, 5% SDS) containing 1 mg/ml Proteinase K. The samples were then subjected to two phenol-chloroform extractions and one chloroform:isoamylalcohol (24:1) extraction, followed by ethanol precipitation.

Cell line DNA (8 ug) was digested with EcoRI, size-fractionated on 1% agarose gels, transferred onto nylon filters, and hybridized to ^{32}P randomly labeled probes (Feinberg and Vogelstein, 1983). The hybridization conditions have been described elsewhere (Ibson et al., 1987). Autoradiography of the membranes was performed at -70°C for 1-3 days using Amersham Hyperfilm-MP.

RNA isolation and northern blot analysis

RNA was isolated from log phase cultures of the cell lines and subjected to northern blot analysis as described by Hensel et al. (1990). The hybridization conditions were the same as those described above in the Southern blot analysis section.

Duplex PCR analysis to detect homozygous deletions of the p16 gene

A 20 ul reaction mixture overlaid with a drop of mineral oil contained a final concentration of 200 ng of genomic DNA, PCR buffer supplied by Promega (10 mM tris-HCL, pH 8.3; 5 mM KCl; 0.1% gelatin); 200 uM dNTP's; 1.5 mM $MgCl_2$; and 0.06 units ul^{-1} Taq polymerase. For amplification of a single locus, 0.2 uM of either the C5.1 (p16) primers or D9S199 primers were used, and for amplification of both loci by multiplex PCR, 0.2 uM of the C5.1 (p16) primers and 0.25 uM of the D9S199 primers were used. Following an initial denaturation period of 7 minutes at 95°C, the DNA was subjected to 20 cycles of amplification consisting of denaturation for 1 minute at 94°C, annealing for 1 minute, with a starting temperature of 55°C and decreasing by 0.5°C per cycle for the first 20 cycles, and elongation for 1 minute at 72°C. The final 10 cycles consisted of denaturation for 1 minute at 94°C, annealing for 1 minute at 45°C and elongation for 1 minute at 72°C followed by a final elongation step of 10 minutes at 72°C. The PCR products were subjected to electrophoresis on a 3% nusieve gel, stained with ethidium bromide and visualized with ultraviolet (uv) light.

DNA analysis by Methylation-specific PCR (MSP)

DNA samples were modified with sodium bisulfite according to the method of Herman et al. (1996), and precipitated with ammonium acetate (3M final concentration) and two volumes of ethanol. The resulting templates were subjected to PCR using oligonucleotides designed from the promoter of the p16 gene (Herman et al., 1996) specific for wildtype, methylated or unmethylated DNA. A 20 ul reaction mixture overlaid with a drop of mineral oil contained a final concentration of 50 ng of genomic DNA, 120 ng of each oligonucleotide, PCR buffer supplied by Promega (10 mM tris-HCL, pH 8.3; 5 mM KCl; 0.1% gelatin); 200 uM dNTPs; 1.5 mM $MgCl_2$; and 0.06 units ul^{-1} Taq polymerase (added once the reaction temperature reached 95°C). The DNA was subjected to 35 cycles of amplification consisting of denaturation for 0.5 minutes at 94°C, annealing for 0.5 minutes at 60°C (for unmethylated-specific oligonucleotides) or 65°C (for wildtype- and methylated-specific oligonucleotides), and elongation for 0.5 minutes at 72°C, followed by a final elongation step of 10 minutes at 72°C. The PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized with uv light.

Infection of breast cancer cell lines with adenoviral constructs

Dilutions of the Ad-p16 and Ad-GFP viral supernatants were added to logarithmic phase cultures of cells growing in 10 cm plates, to give final multiplicity's of infection (moi's) of 0 and 50. Following incubation at 37°C for 40 hours, the cells were harvested and analyzed both for the expression of cell cycle regulatory proteins, by immunoblottting, and cell cycle distribution by FACS.

Transfection of breast cancer cell lines with Tet plasmids

For both transient and stable transfections, cells were grown in 35 mm plates until they reached 50-60% confluence. At this time, 2.4 ug of each plasmid(s) was mixed with a total of 12 ul of lipid per well in serum-free medium, and incubated at room temperature for 15 minutes. The cells were then washed with 1 ml of serum-free medium and overlaid with the DNA/lipid mixture. After 5 hours at 37°C, the DNA/lipid mixture was removed and replaced with medium supplemented with 10% serum, (and the appropriate antibiotic if any), plus or minus 1 ug/ml doxycycline. For transient transfection, the cells were grown for a further 24 hours at 37°C and harvested for p16 protein analysis. For stable transfections, the cells were grown for a further 48 hours then selected for growth in medium supplemented with 500 ug/ml G418 (for the isolation of pUDH15-1 single stable transfectants) or 500 ug/ml G418 plus 500 ug/ml zeocin (for the isolation of pUDH15-1/pTET-SPLICE-p16 double stable transfectants) plus or minus 1 ug/ml doxycycline. Individual antibiotic-resistant colonies were isolated using sterile cloning rings (Bellco) and cultured in selection media for further analysis.

Identification of pUDH15-1 and p16 stable transfectants by PCR analysis

An approximately 50% confluent $25cm^2$ flask of each clone was vigorously shaken to detach cells undergoing mitosis. These cells were collected by centrifugation and lysed in a solution of 10 mM Tris HCL pH 8.7, 50 mM KCl, 1.2 mM $MgCl_2$, 0.01% gelatin, 0.45% NP-40, 0.45% Tween-20 and 10 mg/ml proteinase K at 55°C for 60 minutes. Five microliters of each of the resulting cellular lysates were subjected to PCR analysis using oligonucleotide primers corresponding to the appropriate plasmid in the following manner: A 20 ul reaction mixture overlaid with a drop of mineral oil contained 5 ul genomic DNA, 0.2 uM of each primer, PCR buffer supplied by Promega (10 mM tris-HCL, pH 8.3; 5 mM KCl; 0.1% gelatin); 200 uM dNTP's; 1.5 mM $MgCl_2$; and 0.06 units ul^{-1} Taq polymerase. Following an initial denaturation period of 7 minutes at 95°C, the DNA was subjected to 30 cycles of amplification consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 52°C (for pUDH15-1reactions) and 58°C (for p16 reactions), elongation for 1 minute at 72°C, and a final elongation step of 10 minutes at 72°C. The PCR products were subjected to electrophoresis on a 2 % agarose gel, stained with ethidium bromide and visualized with ultraviolet (uv) light.

Soft agar analysis

Five milliliters of a mixture containing 0.4% agar, 10% serum, 1 X MEM medium, 500 ug/ml G418, 500 ug/ml zeocin, plus or minus 1 ug/ml doxycycline (previously incubated at 45°C) were added to each of 64 60 mm plates, and allowed to harden at room temperature. Each of the transfectants, that had been grown in the presence or absence of 1 ug/ml doxycycline in two 75 cm^2 flasks for 7 days, were harvested and counted. The cells were resuspended in MEM medium supplemented with 10% serum to 1.25 X 10^4 cells/ml, and 4 ml of each suspension added to each of two tubes containing 6 ml of the above agar/medium mixture. After mixing, 2.5 ml of the cell-agar suspension were plated on to 8 of the 60 mm plates containing a base layer of

agar/medium. The parent cell line (MCF-7 or ZR75.1) was also grown in the presence and absence of doxycycline for 7 days and subject to soft agar analysis in the absence of G418 and zeocin selection.

Nude mice tumorigenicity assays

Female athymic nude mice (NCI) of approximately 5-7 weeks of age, were implanted subcutaneously between the right shoulder and neck, with a pellet containing 1.7 mg of 17 B-Estradiol (Innovative Research of America). After 4-7 days, replicate mice were injected subcutaneously into the left shoulder with (a) the MCF-7 parent cell line, (b) the MCF-7/15-1/p16#69 transfectant (that had been cultured in the absence of doxycycline, and expressed moderate levels of p16 protein), and (c) the MCF-7/15-1/p16#13 transfectant (that had been cultured in the absence of doxycycline, and expressed high levels of p16 protein), at 10^7 cells per mouse. The mice were monitored biweekly for signs of tumor growth over an 8 week period.

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PUBLICATIONS

Abstracts

Abnormalities in the Expression of Cell Cycle Regulatory Proteins in Breast Cancer Cell Lines and Tissues.
Presented at: Keystone Symposium on Molecular and Cellular Biology: Genetics of Human Cancer: Pathogenesis and Diagnosis, January 1997.

Abnormalities in the Expression of Cell Cycle Regulatory Proteins Cyclin D1, Retinoblastoma Protein and Multiple Tumor Suppressor 1 in Breast Cancer.
Presented at: Department of Defense Breast Cancer Research Program Meeting: Era of Hope, October 1997.

Enhanced Cyclin D1 Protein Stability and p16 Methylation in Breast Cancer.
Presented at: Keystone Symposium on Molecular and Cellular Biology: The Cell Cycle, March 1998.

Manuscripts in Preparation

Concomitant Inactivation of p16 and Overexpression of Cyclin D1 protein in Breast Tumorigenesis. Effect of Expression of p16 protein and Anti-sense Cyclin D1 on Growth and Tumorigenic Properties. Todd, M.C., Schauer, I.E., Sclafani, R.A. and Langan, T.A.

Overexpression of p16 and Cyclin D1 proteins in Rb-positive Breast Tumors and Cell Lines. Indications of an Unusual Mechanism for Overcoming Cell Cycle Regulatory Control. Todd, M.C., Sclafani, R.A., Franklin, W. and Langan, T.A.

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APPENDIX

ROLE OF CHANGES IN THE EXPRESSION OF CYCLINS AND RETINOBLASTOMA PROTEIN IN THE DEVELOPMENT OF BREAST CANCER

PRINCIPAL INVESTIGATOR: Thomas A. Langan, Ph.D.

Award No. DAMD17-94-J-4481

Figure 1 Expression of cell cycle regulatory proteins in normal and tumor breast epithelial cell lines. Extracts were prepared from the indicated cell lines and subjected to SDS/PAGE. Following transfer to Immobilon P membranes they were probed with a panel of 15 antibodies. A representative selection of western blots is shown. The normal breast epithelial cell lines included in the panel were MCF-12A and HBL-100. nt denotes not tested.

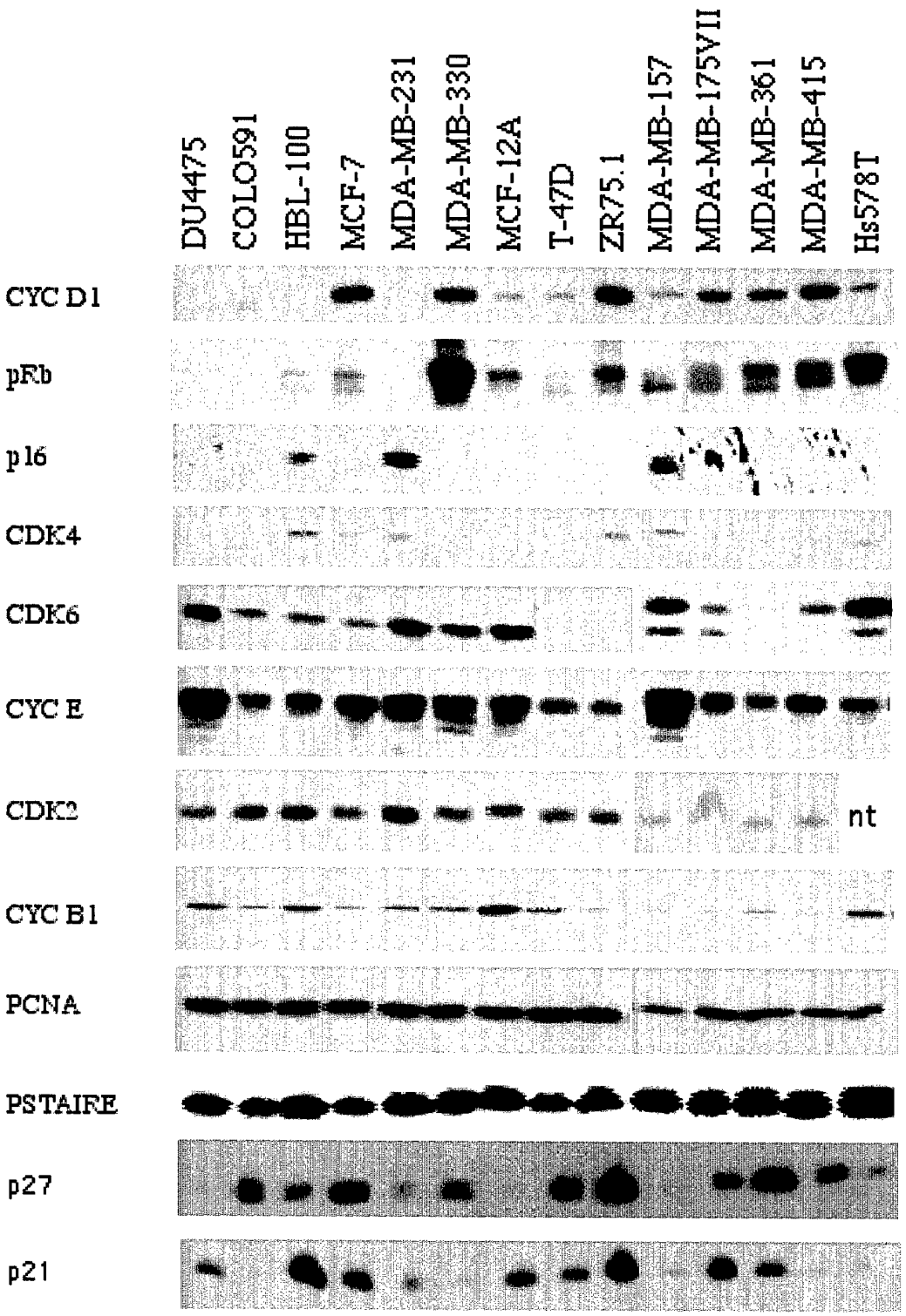

DU4475
COLO591
HBL-100
MCF-7
MDA-MB-231
MDA-MB-330
MCF-12A
T-47D
ZR75.1
MDA-MB-157
MDA-MB-175VII
MDA-MB-361
MDA-MB-415
Hs578T
CYC D1
pRb
p16
CDK4
CDK6
CYC E
CDK2
nt
CYC B1
PCNA
PSTAIRE
p27
p21

Figure 2 The half-life of cyclin D1 protein is extended in the breast cancer cell line, ZR75.1. Log phase cultures of the indicated cell lines were treated with 0.1 mg/ml cycloheximide, harvested at the time intervals shown and extracts prepared. Following SDS/PAGE, the extracts were transferred to Immobilon P membranes and probed with an antibody to cyclin D1.

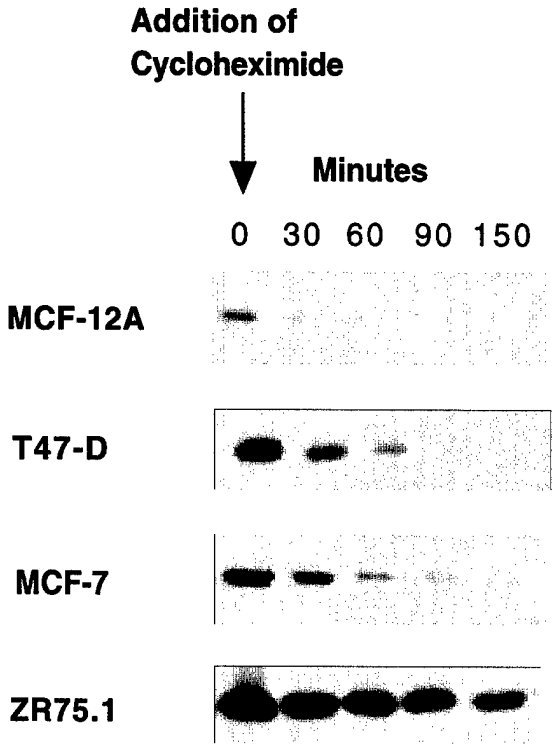

Addition of
Cycloheximide
Minutes
0 30 60 90 150
MCF-12A
T47-D
MCF-7
ZR75.1

Figure 3 Effect of serum-deprivation upon cyclin D1 protein expression in pRb-positive breast cancer cell lines. The normal breast epithelial cell line, MCF-12A and each of the nine breast cancer cell lines that express cyclin D1 were grown in the absence of serum for 1, 2 and 3 days respectively. Extracts were then prepared, subjected to SDS-PAGE, transferred to Immobilon P membranes and probed with an antibody to cyclin D1. Two representative breast cancer cell lines are shown, as is the normal breast epithelial cell line, MCF-12A.

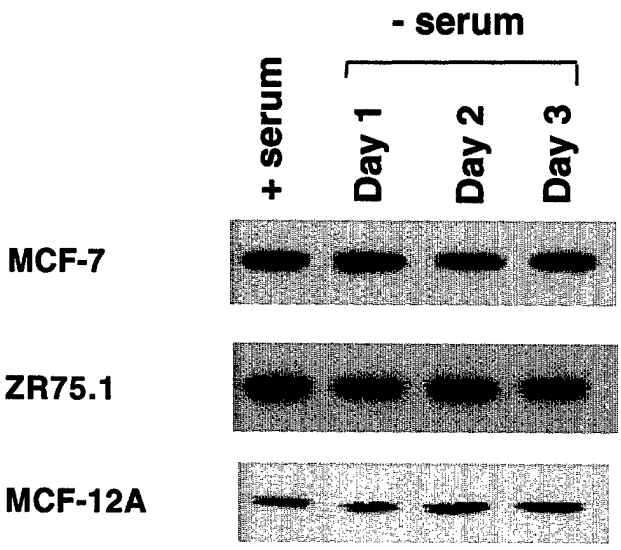

- serum
+ serum
Day 1
Day 2
Day 3
MCF-7
ZR75.1
MCF-12A

Figure 4 Effect of cellular confluence upon cyclin D1 expression. The normal breast epithelial cell line, MCF-12A, and three breast cancer cell lines (that showed 10-fold overexpression of cyclin D1 protein), MCF-7, MDA-MB-330 and ZR75.1, were grown to 50% confluence (lane 1), 85% confluence (lane 2) and 100% confluence (lane 3). Extracts from each of the cultures were subjected to SDS/PAGE, transferred to Immobilon P, and probed with an antibody to cyclin D1. Only the MCF-12A cells showed a reduction in cyclin D1 expression to barely detectable levels in the 100% confluent culture.

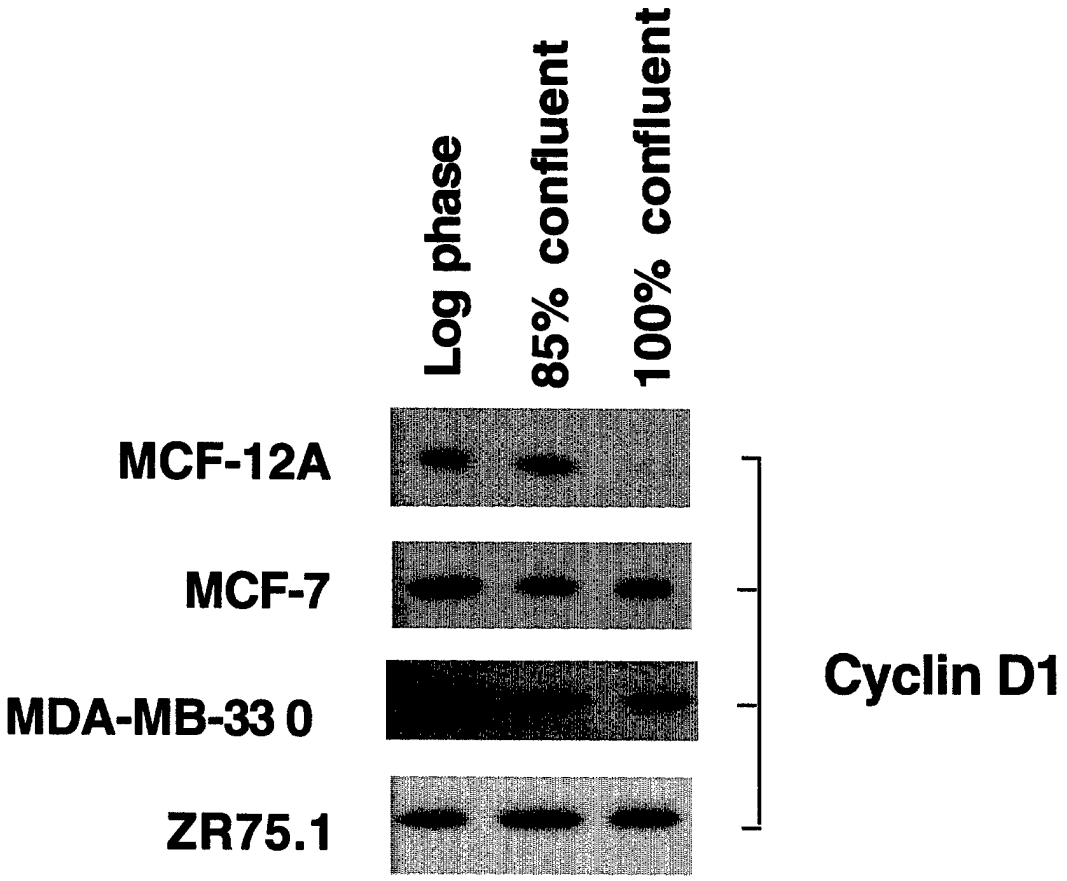

Log phase
85% confluent
100% confluent
MCF-12A
MCF-7
MDA-MB-33 0
ZR75.1
Cyclin D1

Figure 5 PCR analysis of p16 gene homozygous deletion in breast cancer cell lines. Oligonucleotide primers used were: c5.1 (p16) (first lane under each cell line heading); c5.1 (p16) and D9S199 (second lane under each cell line heading); and D9S199 (third lane under each cell line heading). Following amplification the PCR products were electrophoresed on 3% NuSieve gels. The amplicons were visualized with uv following ethidium bromide staining. Four of the cell lines failed to amplify the c5.1 (p16) product whereas all of the cell lines amplified the control D9S199 product. DNA derived from two normal breast cell lines, MCF-12A and HBL-100, a normal breast tissue sample, and a breast cancer cell line, COLO 742 (not described in the analysis of cell cycle protein expression) were included in the panel. The three lanes under the heading "Blanks" contain all of the components of the PCR reaction mixtures minus DNA to indicate the absence of contamination.

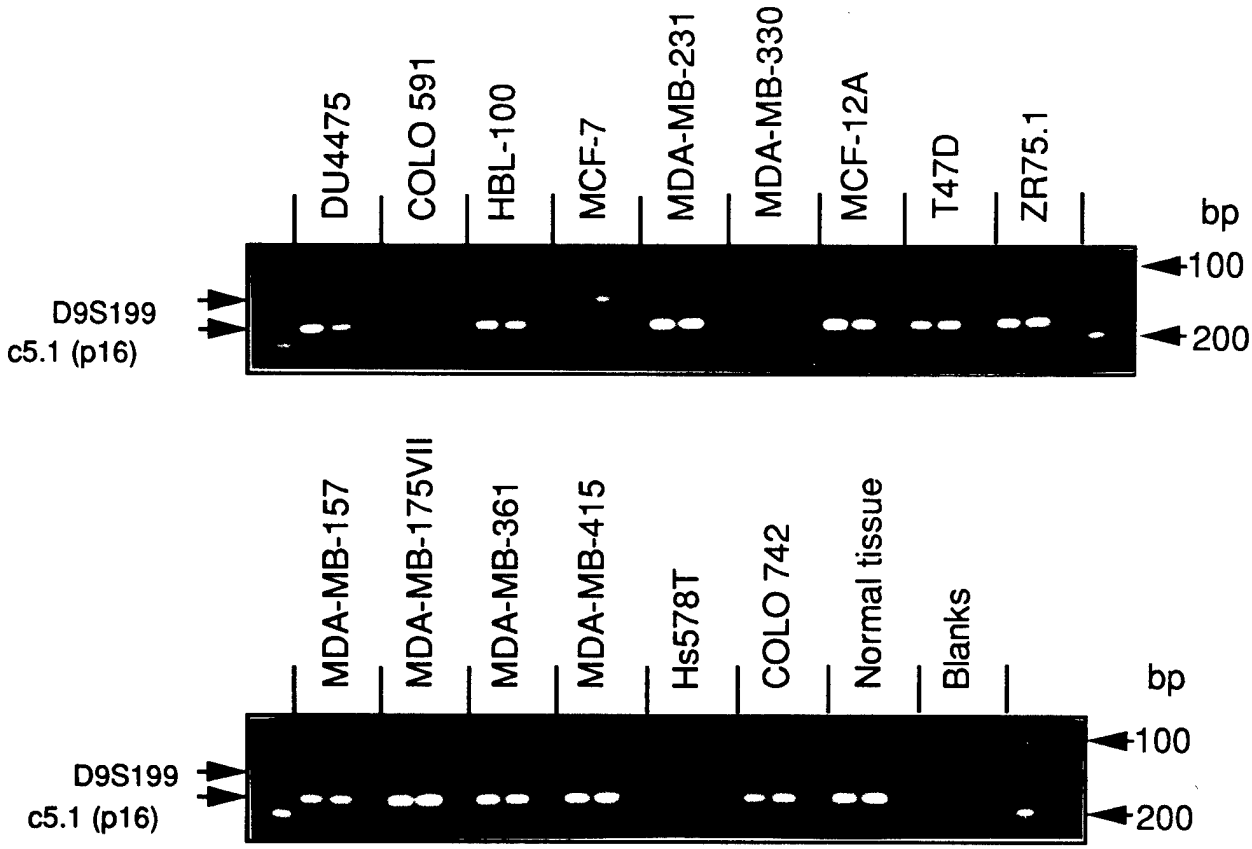

DU4475
COLO 591
HBL-100
MCF-7
MDA-MB-231
MDA-MB-330
MCF-12A
T47D
ZR75.1
bp
100
200
D9S199
c5.1 (p16)
MDA-MB-157
MDA-MB-175VII
MDA-MB-361
MDA-MB-415
Hs578T
COLO 742
Normal tissue
Blanks
bp
100
200
D9S199
c5.1 (p16)

Figure 6 Methylation-specific PCR (MSP) of breast cancer cell lines at the p16 locus. Following modification of cell line DNA with sodium bisulfite (converting all unmethylated cytosine's to uracil), it was subjected to PCR using primers from the promoter of the p16 gene specific for methylated (M) or unmethylated (U) DNA. A pair of primers specific for unmodified (WT) DNA was also used as a control for completeness of the sodium bisulfite reaction. PCR products were electrophoresed on a 2% agarose gel and the amplicons visualized with uv following ethidium bromide staining. Two of the cell lines, DU4475 and T-47D, showed amplification with the primers specific for methylated DNA and one of the cell lines, ZR75.1, showed amplification with the primers specific for both methylated and unmethylated DNA. The normal breast epithelial cell line, MCF-12A was shown to undergo weak amplification with the primers specific for unmethylated DNA on three different occasions. However, the ethidium bromide-stained amplicon is not clearly visible in this figure. Modified DNA derived from a breast cancer cell line, COLO 742 (not described in the analysis of cell cycle protein expression) and a lung cancer cell line, NCI H 157, wee also included in the panel as controls for unmethylated p16. The three lanes labeled "Blanks" contain all of the components of each of the three PCR mixtures minus DNA to indicate the absence of contamination.

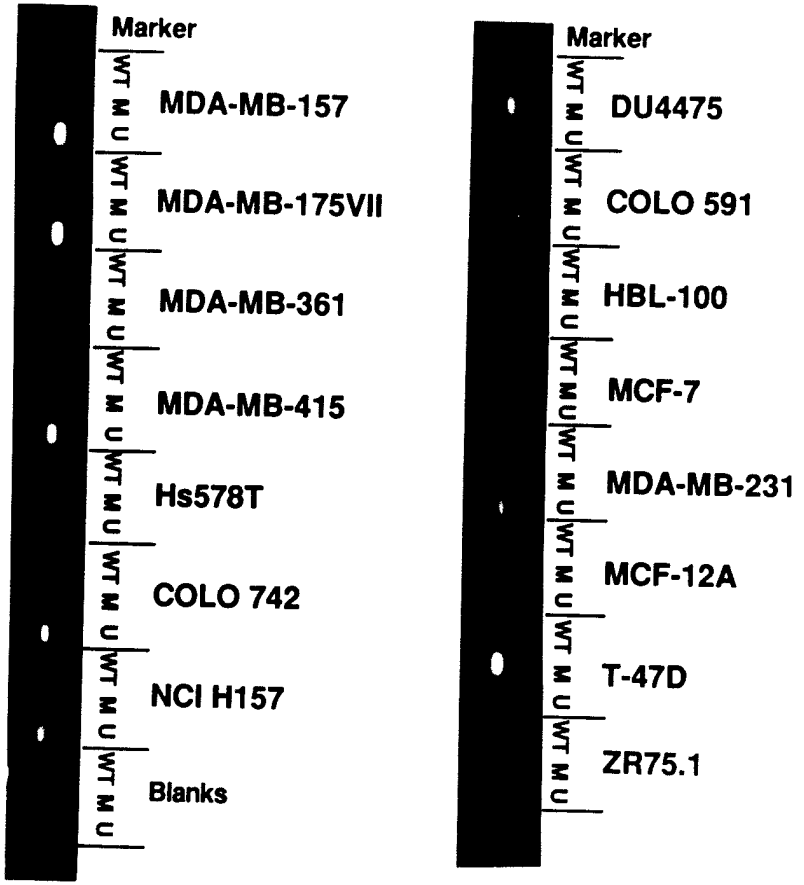

Marker
WT M U
MDA-MB-157
WT M U
MDA-MB-175VII
WT M U
MDA-MB-361
WT M U
MDA-MB-415
WT M U
Hs578T
WT M U
COLO 742
WT M U
NCI H157
WT M U
Blanks
Marker
WT M U
DU4475
WT M U
COLO 591
WT M U
HBL-100
WT M U
MCF-7
WT M U
MDA-MB-231
WT M U
MCF-12A
WT M U
T-47D
WT M U
ZR75.1
WT M U

Figure 7 Immune complex kinase analysis of MCF-7 lysates. Following immunoprecipitation of MCF-7 lysates with cyclin D1, CDK2, CDK6, CDK4 and normal rabbit serum (as a negative control), the resulting complexes were washed and assayed for kinase activity using GST-Rb-(C) as substrate. The kinase reactions were stopped with SDS/PAGE loading buffer, boiled, subjected to SDS/PAGE and exposed for autoradiography.

cyclin D1

CDK2

CDK6

CDK4

rabbit serum

GST-Rb-(C)

Figure 8 Cyclin D1 and Rb protein expression in matched pairs of normal and tumor breast tissues. Protein extracts were prepared from breast tissues, subjected to SDS/PAGE, transferred to Immobilon P membranes, and probed with antibodies to cyclin D1, Rb and PSTAIRE. "T" and "N" suffixes designate tumor and normal tissue samples, respectively.

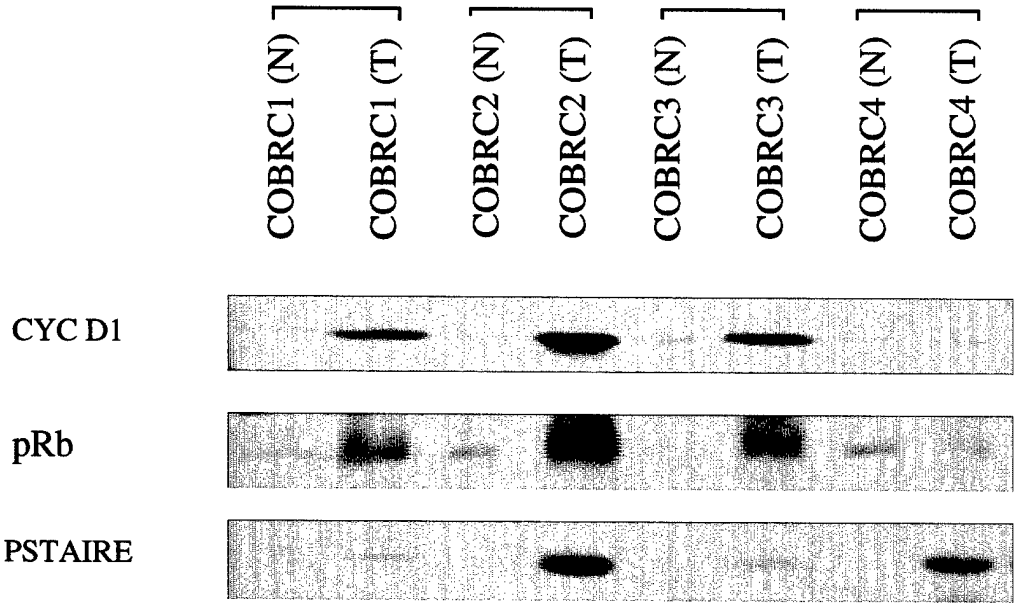
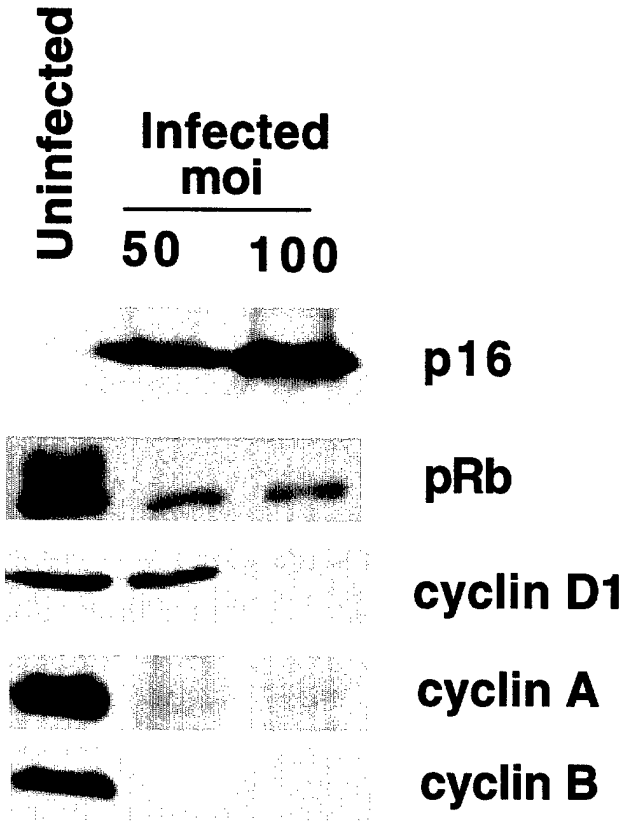

COBRC1 (N)
COBRC1 (T)
COBRC2 (N)
COBRC2 (T)
COBRC3 (N)
COBRC3 (T)
COBRC4 (N)
COBRC4 (T)
CYC D1
pRb
PSTAIRE

Figure 9 Infection of breast cancer cells with an adenoviral-p16 construct, Ad-p16, results in a high level of p16 expression and G1-phase arrest. Logarithmic phase MCF-7 cells were infected with Ad-p16 at an moi of 50 and 100, or left uninfected. After 42 hours at 37°C, cells were harvested for FACS and immunoblot analyses. Uninfected cells showed the expected cell cycle distribution and expression of cyclin proteins for a logarithmic population, in addition to the lack of p16 expression and overexpression of cyclin D1 protein characteristic of this cell line. Infected cells, by contrast, expressed extremely high levels of p16 protein that resulted in the degradation of cyclin D1 protein, a shift of Rb to the hypophosphorylated form and G1 accumulation. The latter is illustrated by the significant loss of expression of the S and G2/M phase-specific cyclins, A and B, respectively.



% G1	61	94	95
% S	29	6	3
% G2/M	10	0	2

Figure 10 Transient transfection of MCF-7 (panel A) and ZR75.1 (panel B) with p16. Cells were transfected with DNA and grown in the presence (+) or absence (-) of doxycycline for 24 hours. 100 μg of each extract were then subjected to SDS/PAGE, transferred to nitrocellulose and probed with antibodies to p16 and PSTAIRE (as a loading control). Lanes 1(+) and 1(-), untransfected cells; lanes 2(+) and 2(-), transfected with pTET-SPLICE-p16 and pUDH15-1; lanes 3(+) and 3(-), transfected with pTET-SPLICE-p16 only; lane 4, HBL-100 (positive control for p16).

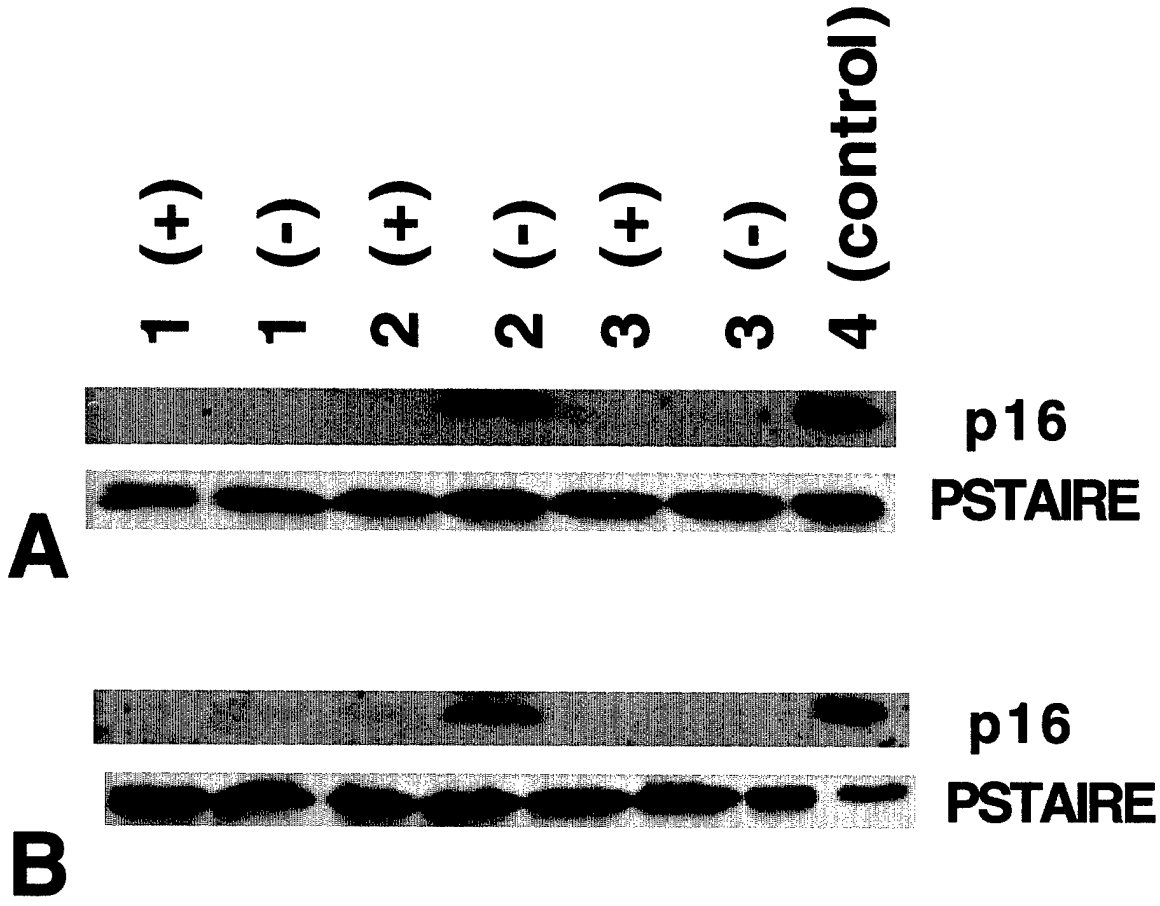
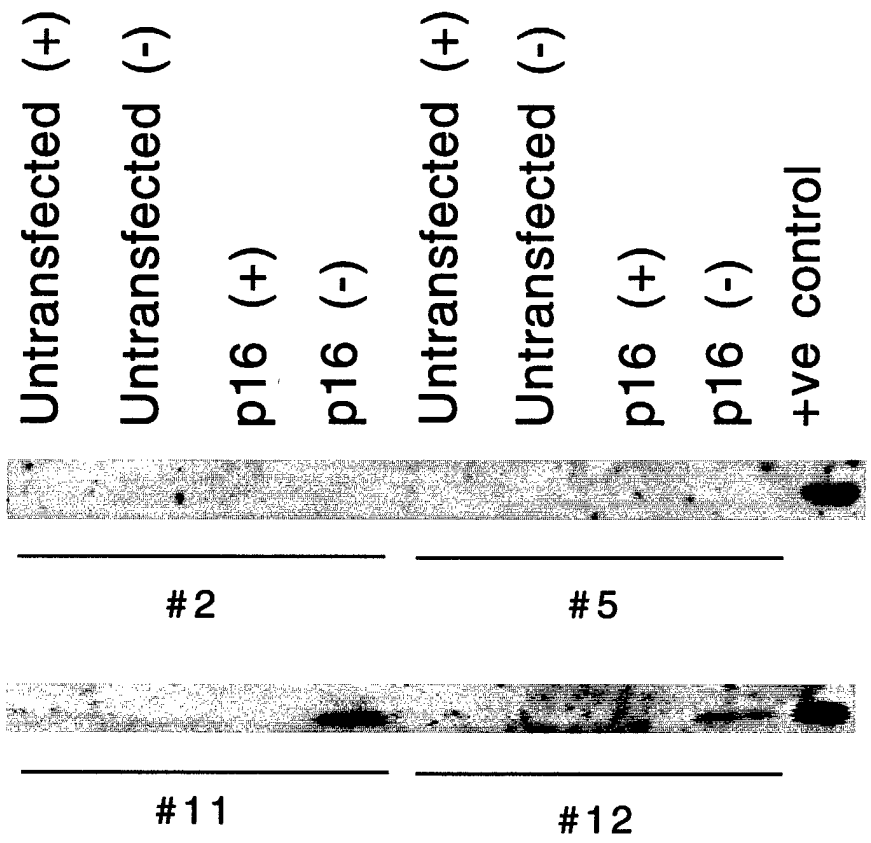
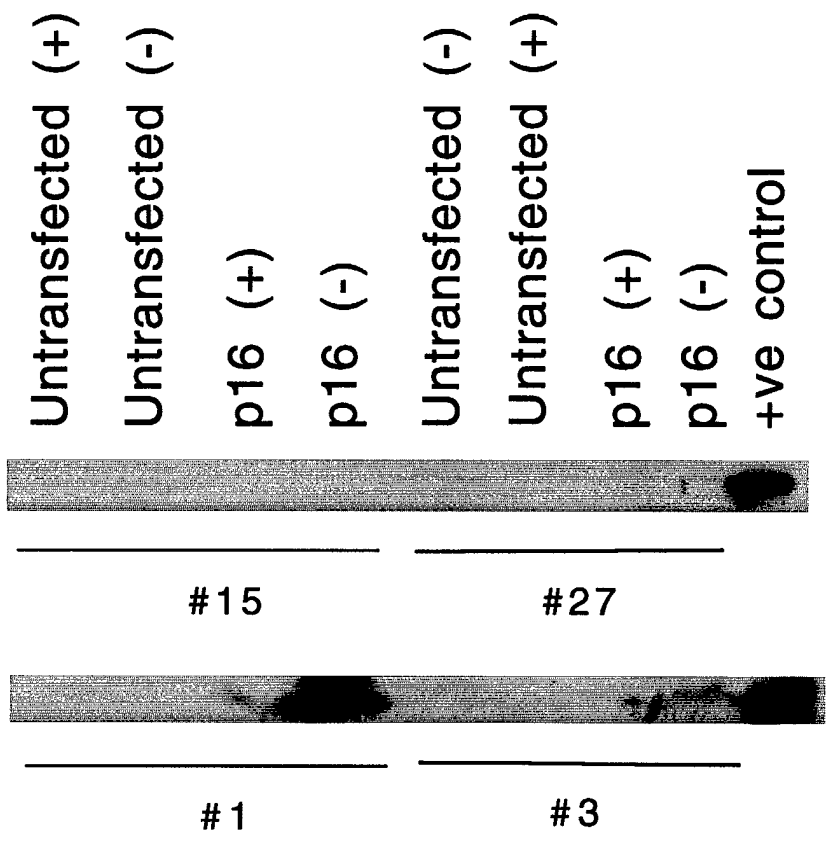

1 (+)
1 (-)
2 (+)
2 (-)
3 (+)
3 (-)
4 (control)
p16
PSTAIRE
A
p16
PSTAIRE
B

Figure 11 Induction of p16 expression by tTA. MCF-7 and ZR75.1 clones stably transfected with the pUDH15-1 plasmid encoding the tTA transcriptional regulator were subjected to transient transfection with pTET-SPLICE-p16, and grown in the presence (+) or absence (-) of doxycycline for 24 hours. The resulting transfectants showed a wide range of p16 expression levels in the absence of doxycycline, a representative selection of which are shown in the figure.

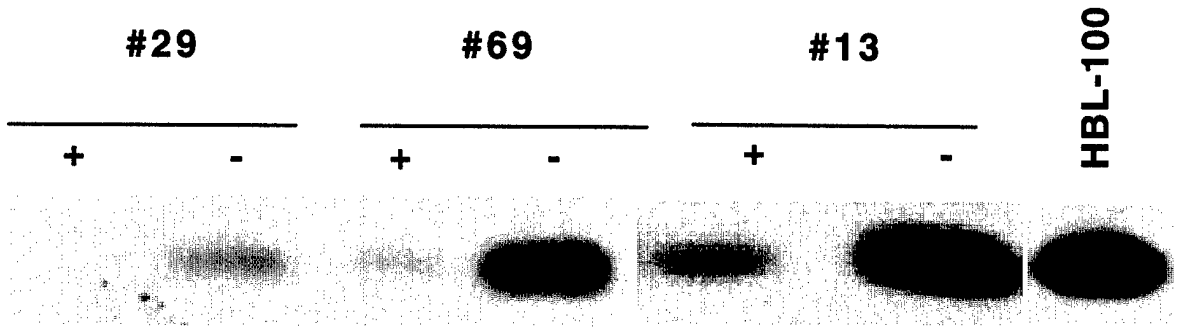


MCF-7/15-1

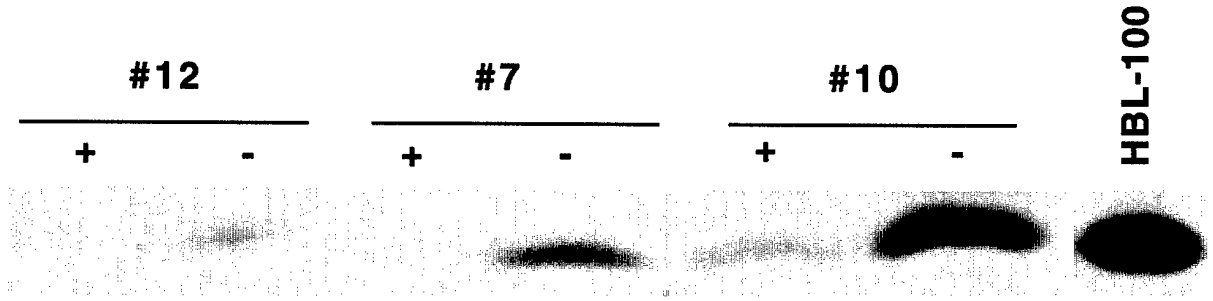


ZR75.1/15-1

Figure 12 Induction of p16 expression in MCF-7 and ZR75.1 clones stably transfected with pUDH15-1 and pTET-SPLICE-p16. MCF-7 and ZR75.1 "double stable" clones were grown in the presence and absence of doxycycline for 7 days then subjected to immunoblot analysis using an antibody to p16. The clones exhibited a range of p16 expression following the removal of doxycycline (and in some cases, a low level of expression in the presence of doxycycline). Representative clones expressing low, moderate and high levels of p16 induction (relative to HBL-100 that exhibits overexpression of p16) are shown in the figure.

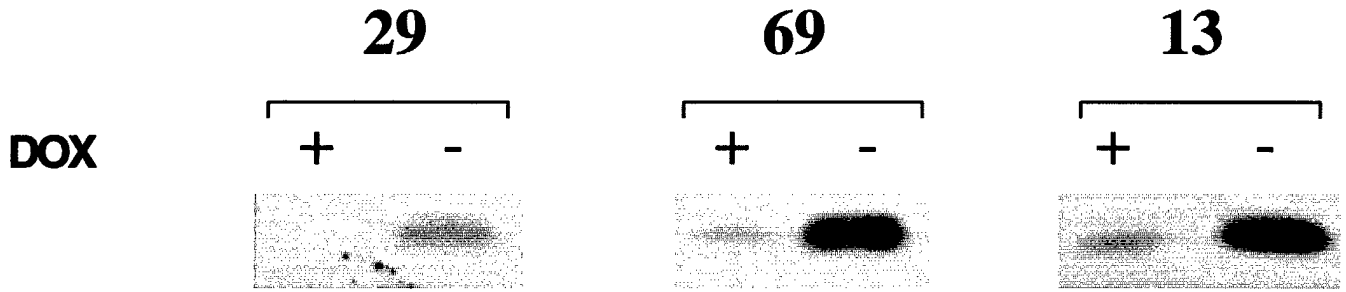


MCF-7/15-1/p16



ZR75.1/15-1/p16

Figure 13 G1 accumulation following doxycycline-induced p16 expression in MCF-7 breast cancer cell line transfectants. Three MCF-7 "Tet" clones, MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13, were grown in the presence or absence of doxycycline for 7 days prior to immunoblot and cell cycle distribution analyses. The induction of moderate and high expression of p16 in the absence of doxycycline (in clones #69 and #13, respectively), correlated with a significant accumulation of cells in G1 phase.



% G1	67	71	66	87	63	87

Figure 14 Comparison of p16 promoter methylation versus p16 protein expression in normal and tumor breast tissue pairs. A. DNA derived from microdissected normal (N) and tumor (T) breast tissue cells was subjected to amplification with primers specific for wildtype (WT) (unmodified), methylated (M) and unmethylated (U) p16 promoter sequences. All of the normal and tumor tissue pairs underwent amplification with the primers specific for unmethylated DNA, a representative selection of which are shown in the figure. The breast cancer cell lines, MDA-MB-175VII and T-47D, were included as controls for unmethylated (U) and methylated (M) DNA respectively; and the breast cancer cell line, MCF-7, which is homozygously deleted for the p16 gene, was included as a negative control for amplification. Amplification with primers specific for WT DNA indicated the presence of residual unmodified DNA. B. Immunoblot analysis of p16 protein expression in representative pairs of normal and tumor tissue extracts. All of the tumors expressed equivalent or abnormally elevated levels of p16 protein, relative to their normal tissue counterparts. Two tissue pairs including COBRC13 (N) and (T), indicated in the figure, expressed equivalent, but barely detectable levels of p16 protein. All but one of the p16-positive tumors, COBRC4 (T), also expressed Rb protein.

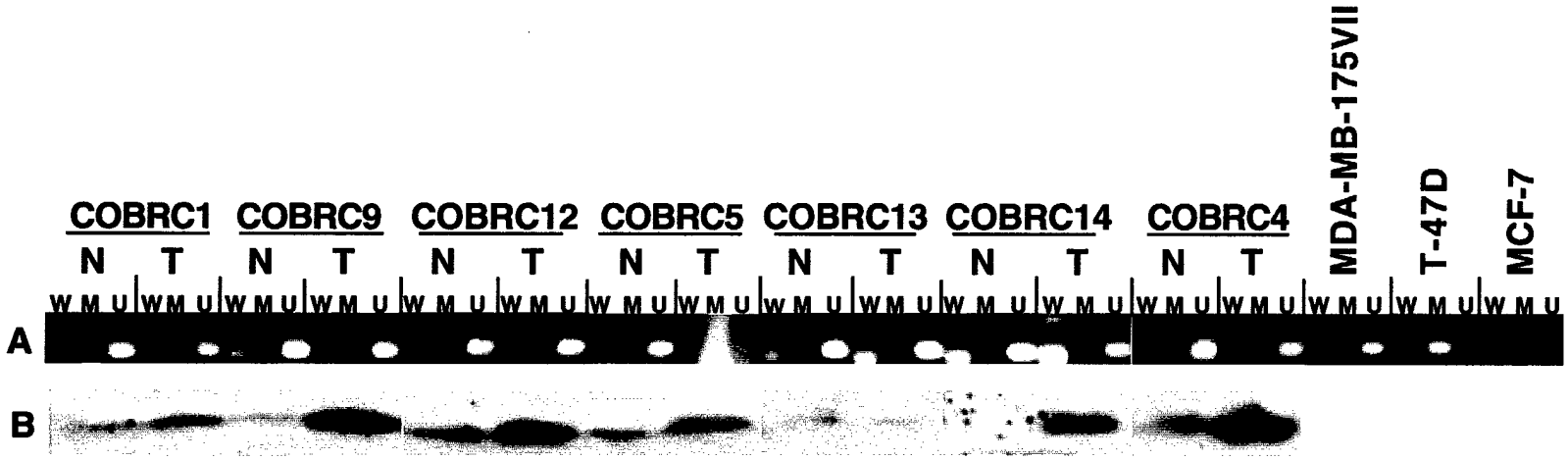

COBRC1
COBRC9
COBRC12
COBRC5
COBRC13
COBRC14
COBRC4
N T N T N T N T N T N T N T
W M U W M U W M U W M U W M U W M U W M U W M U W M U W M U W M U W M U W M U W M U
MDA-MB-175VII
T-47D
MCF-7
A
B

Figure 15 Expression of p16, Rb and cyclin D1 proteins in matched normal and tumor breast tissue extracts. Equivalent amounts of normal and tumor tissue extracts were immunoblotted with antibodies to p16, Rb and cyclin D1 proteins. The majority of tumors expressed Rb protein, in addition to p16 and cyclin D1 proteins. Levels of p16 protein expression in the Rb-positive tumors were at least equivalent to, and frequently in excess of, that demonstrated by the normal tissue counterparts. Representative tissue pairs showing the range of p16 protein levels detected, are indicated in the figure. With the exception of COBRC4 (T) which did not express Rb protein, all of the tumors expressed significantly higher levels of Rb protein than their normal tissue counterparts. To visualize both the hypophosphorylated and hyperphosphorylated species of Rb protein in the tumor tissues, we have displayed very short exposures of the Rb immunoblots. This accounts for the correspondingly low (and in the case of COBRC9 (N), barely detectable) levels of Rb protein in the normal tissue lanes.

DU4475
COLO591
HBL-100
MCF-7
MDA-MB-231
MDA-MB-330
MCF-12A
T-47D
ZR75.1
MDA-MB-157
MDA-MB-175VII
MDA-MB-361
MDA-MB-415
Hs578T
MDA-MB-134VI
COLO 742
pRB
CYC D1
p16

Figure 16 Expression of Rb, p16 and cyclin D1 proteins in normal and tumor breast epithelial cell lines. Extracts derived from each of 14 breast cancer cell lines, and two normal (MCF-12A and HBL-100) breast epithelial cell lines were assessed for the level of three critical G1/S phase regulatory proteins. The two new cell lines added to the panel are shown at the right. Immunoblotting for p16 was done with the more sensitive antibody from Oncogene. For the 12 cell lines on the left, the Rb and cyclin D1 data from Figure 1 is reproduced for comparison. Whereas, the majority of cell lines showed coexpression of Rb and cyclin D1 proteins, with concomitant loss of p16 expression, three cell lines (MDA-MB-157, MDA-MB-415 and COLO 742) demonstrated coexpression of Rb, p16 and cyclin D1 proteins.

DU4475
COL0591
HBL-100
MCF-7
MDA-MB-231
MDA-MB-330
MCF-12A
T-47D
ZR75.1
MDA-MB-157
MDA-MB-175VII
MDA-MB-361
MDA-MB-415
Hs578T
MDA-MB-134VI
COLO 742
pRB
CYC D1
p16

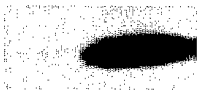
Figure 17 MDA-MB-157 and COLO 742 cells are completely insensitive to high levels of ectopic functional p16 protein expression. Logarithmic phase MDA-MB-157, COLO 742, MCF-7 and HBL-100 cells were infected with Ad-p16 at an moi of 50. Only the p16-negative cell line, MCF-7, showed a G1-phase arrest in response to high levels of ectopic p16 expression. By contrast, both uninfected and Ad-p16-infected cells of the SV40-transformed (functionally Rb negative) HBL-100 cell line, and the Rb/p16-positive MDA-MB-157 and COLO 742 cell lines, exhibited virtually identical logarithmic cell cycle distribution profiles.

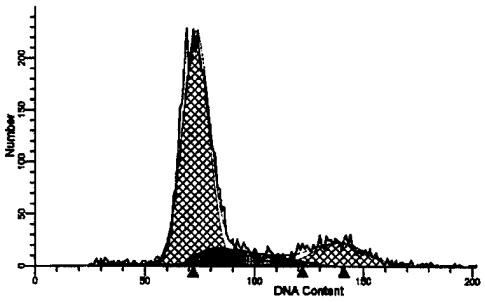
Uninfected

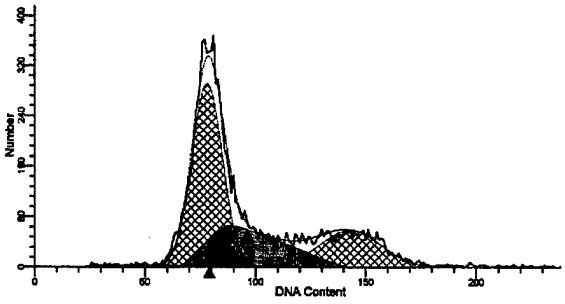
Ad-p16 moi = 50

MDA-MB-157

Un Inf

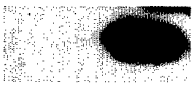


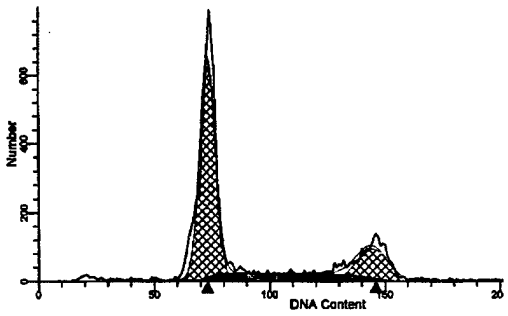


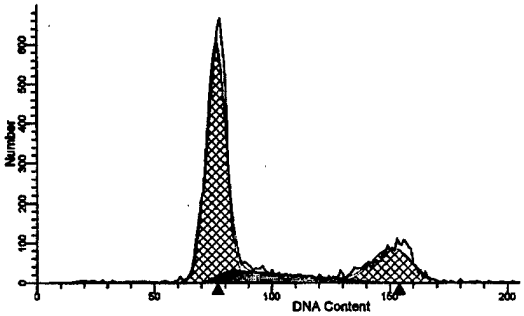


COLO 742

Un Inf

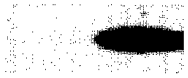


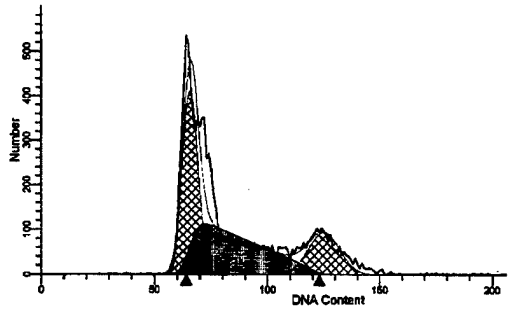


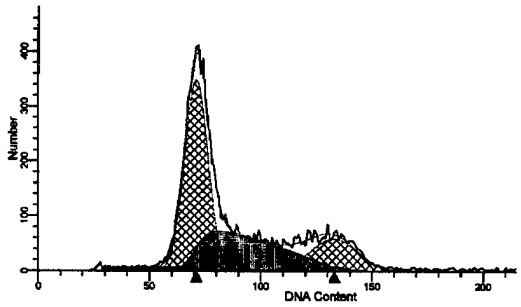


HBL-100

Un Inf

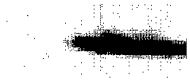


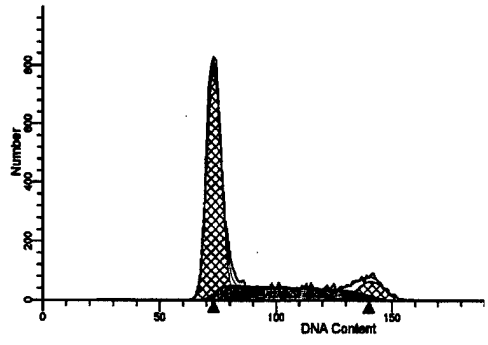




MCF-7

Un Inf





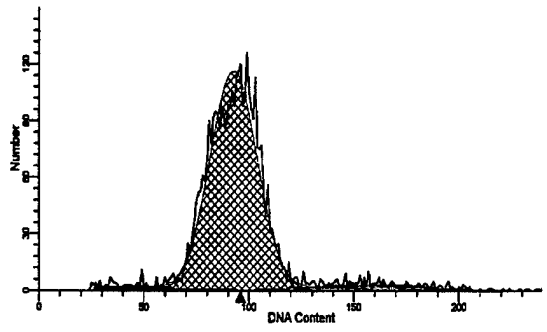
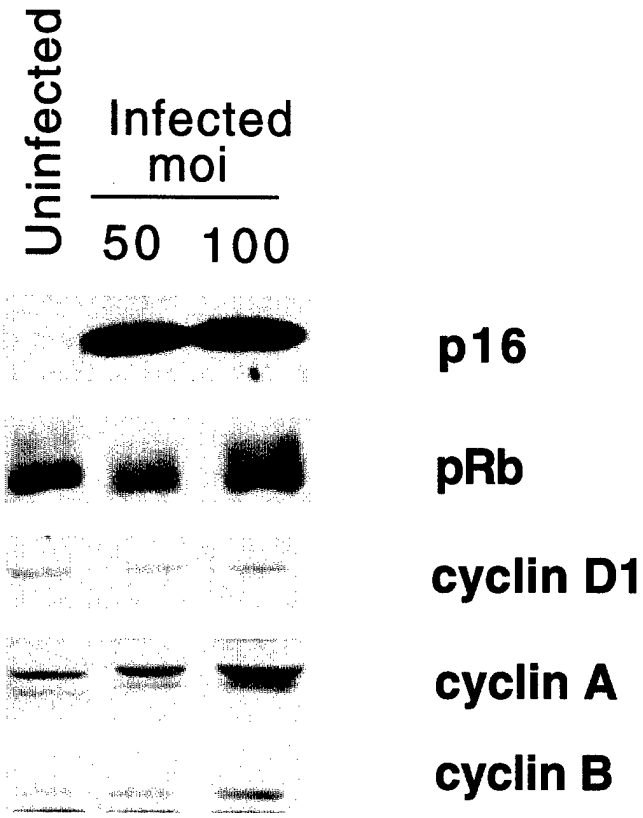


Figure 18 MDA-MB-157 cells are refractory to high levels of ectopic functional p16 protein expression. Logarithmic phase MDA-MB-157 cells were infected with Ad-p16 at an moi of 50 and 100, giving rise to exceptionally high levels of p16 protein expression. However, the infected cells continued to grow unabated, as indicated by the lack of both G1 phase accumulation and significant alterations in G1/S regulatory protein expression.



%G1	70	52	56
% S	15	27	32
% G2/M	15	21	12

Table 1 Expression of cyclin D1, Rb and p16 proteins in breast cancer cell lines.

Cell line	Histology	Rb protein	Cyclin D1 protein	p16 protein
Non-tumor cell lines				
MCF-12A	Normal epithelial	+	+	-
HBL-100	Normal epithelial	-*	-	+
Tumor cell lines				
MCF-7	Adenocarcinoma	+	+++++	-
MDA-MB-330	Carcinoma	+	+++++	-
ZR75.1	Carcinoma	+	+++++	-
MDA-MB-175VII	Ductal carcinoma	+	+++	-
MDA-MB-361	Adenocarcinoma	+	+++	-
MDA-MB-415	Adenocarcinoma	+	+++++	-
T47-D	Ductal Carcinoma	+	+	-
Hs578T	Ductal carcinoma	+	+	-
MDA-MB-157	Medula carcinoma	+	+	+
DU4475	Carcinoma	-/+	-/+	-
COLO 591	not known	-	-	-
MDA-MB-231	Adenocarcinoma	-	-	+

The presence or absence of Rb and p16 expression is indicated by a (+) or a (-) respectively. *Although HBL-100 expresses Rb protein the latter is complexed to SV40 T Ag and is therefore non-functional. The approximate levels of cyclin D1 protein (relative to the non-tumor cell line MCF-12A) are indicated as follows: (+++++), 10X; (+++), 5X; (+), 1X (equivalent to MCF-12A); (-/+), barely detectable; (-), undetectable.

TABLE 2

COMPARISON OF CYCLIN D1 GENE AMPLIFICATION AND mRNA AND PROTEIN EXPRESSION IN BREAST CANCER CELL LINES

Cell Line	DNA	mRNA	cyclin D1 protein
MCF-7	3x	2x	10x
MDA-MB-330	2x	2x	10x
ZR75.1	5x	2x	10x
MDA-MB-175VII	3x	2x	5x
MDA-MB-361	3x	2x	5x
MDA-MB-415	10x	5x	10x

Table 3 Summary of alterations in the p16 gene in breast cancer cell lines

		p16 gene		
Cell line	p16 protein	homozygous deletion	methylated promoter	unmethylated promoter
MCF-12A	-			+
HBL-100	+			+
MCF-7	-	+		
MDA-MB-330	-	+		
ZR75.1	-		+	+
MDA-MB-175VII	-			+
MDA-MB-361	-			+
MDA-MB-415	-			+
T-47D	-		+	
Hs578T	-	+		
MDA-MB-157	+			+
DU4475	-		+	
COLO 591	-	+		
MDA-MB-231	+			+

DNA derived from each of the normal and tumor breast epithelial cell lines was analyzed for the homozygous deletion or methylation of the p16 gene using PCR-based assays. Seventy percent of the cell lines that failed to express p16 protein were found to have undergone either the deletion or methylation of the gene.

Table 4 Expression of Rb and cyclin D1 proteins in primary breast cancer tissues

Tissue	Histology	Rb protein	Cyclin D1 protein
COBRC1 (N)	Normal epithelial	+	+
COBRC1 (T)	Ductal carcinoma	+	+++
COBRC2 (N)	Normal epithelial	+	+
COBRC2 (T)	In situ Ductal carcinoma	+	++++
COBRC3 (N)	Normal epithelial	+	+
COBRC3 (T)	Carcinoma	+	+++
COBRC5 (N)	Normal epithelial	+	+
COBRC5 (T)	unknown	+	+++
COBRC6 (N)	Normal epithelial	+	+
COBRC6 (T)	unknown	+	++
COBRC10 (N)	Normal epithelial	+	+
COBRC10 (T)	unknown	+	+++
COBRC7 (N)	Normal epithelial	+	+/-
COBRC7 (T)	unknown	+	+/++
COBRC9 (N)	Normal epithelial	+	+/-
COBRC9 (T)	unknown	+	+/++
COBRC4 (N)	Normal epithelial	+	-/+
COBRC4 (T)	Medulla carcinoma	-/+	-/+
COBRC8 (N)	Normal epithelial	+/-	-
COBRC8 (T)	unknown	-	-

The presence or absence of Rb and p16 expression is indicated by a (+) or a (-) respectively. The levels of cyclin D1 protein are indicated as follows: (++++), very high; (+++), high; (++), moderate; (+), equivalent of matched normal tissue; (-/+), barely detectable; (-), undetectable.

Table 5 Tumorigenicity of breast cancer cell lines

Breast cancer cell line	Tumor diameter (cm)
MCF-7	0.15
MCF-7	0.15
MCF-7	0
ZR75.1	0.5
ZR75.1	0.5
ZR75.1	0.55
MDA-MB-231	1.2
MDA-MB-231	1.4
MDA-MB-321	2.0

Logarithmic phase breast cancer cells were injected in triplicate subcutaneously into the left shoulders of athymic nude mice. The tumors were allowed to grow for a period of 4-weeks at which time the diameters were recorded (as indicated above), and both the MDA-MB-231 and ZR75.1-injected mice euthanized. The mice injected with MCF-7 cells were allowed to live for a further 4 weeks but we observed no further increase in tumor size.

Table 6 Growth of MCF-7 and ZR75.1 "Tet transfectants" in soft agar

	% colony formation[1]	
Cell line/transfectant	(+) doxycycline	(-) doxycycline
MCF-7	100	100
MCF-7/15-1/p16#29	~60	~40
MCF-7/15-1/p16#69	<0.1	<0.1
MCF-7/15-1/p16#13	<0.1	<0.1
ZR75.1	100	100
ZR75.1/15-1/p16#12	<2	<2
ZR75.1/15-1/p16#7	~40-50	<2
ZR75.1/15-1/p16#10	<2	<2
MCF-7/15-1/p16#2[2]	~90	~90
MCF-7/15-1/p16#12[2]	~90	~90

[1]The number of colonies that grew from each of the transfectants plated onto soft agar (in the presence or absence of doxycycline) was expressed as a percentage of the total number of colonies that arose from the corresponding parent cell line.
[2]These two MCF-7 transfectants harbored both the regulator (pUDH15-1) and response (pTET-SPLICE-p16) plasmids, but did not express p16 protein.

Table 7 Tumorigenicity of MCF-7 cells versus MCF-7 "Tet" transfectants expressing p16 protein

Cell line/transfectant	Diameter of tumor (cm)
MCF-7	0.45
MCF-7	0.35
MCF-7	0.30
MCF-7/15-1/p16#69	0
MCF-7/15-1/p16#69	0
MCF-7/15-1/p16#69	0
MCF-7/15-1/p16#13	0
MCF-7/15-1/p16#13	0
MCF-7/15-1/p16#13	0

Female athymic nude mice, implanted with estrogen pellets, were injected, in triplicate, subcutaneously into the shoulder with MCF-7 and each the two p16 "Tet" transfectants, MCF-7/15-1/p16#69 and MCF-7/15-1/p16#13 (at 10^7 cells/mouse). The injection site was monitored biweekly for tumor growth, and at the end of 8 weeks, the diameters of the tumors were measured, as indicated above. Mice injected with transfected MCF-7 cells were monitored for an additional 4 weeks, and still showed no detectable tumors.

Table 8 p16, Rb and cyclin D1 protein expression in breast tumor tissues

Tissue	Rb protein	Cyclin D1 protein	p16 protein
COBRC5 (T)	+	+++	++
COBRC6 (T)	+	++	++
COBRC9 (T)	+	+/++	++++
COBRC12 (T)	+	++++	++++
COBRC14 (T)	+	+++	++++
COBRC1 (T)	+	+++	+
COBRC7 (T)	+	+/++	+
COBRC10 (T)	+	+++	+
COBRC11 (T)	+	+	+
COBRC13 (T)	+	+	-/+*
COBRC15 (T)	+	+	-/+*
COBRC4 (T)	-/+	-/+	+++

Matched pairs of normal and tumor breast tissues were assessed for the expression of p16, Rb and cyclin D1 proteins, by immunoblot analysis. The levels expressed by each of the tumors (relative to their normal tissue counterparts) are indicated in the table. Tumors representing the five new tissue pairs are designated COBRC11-15. A (+) or a (-) indicated the presence or absence of Rb protein expression. The levels of p16 and cyclin D1 proteins expressed by the tumors are indicated as follows: (-), undetectable; (-/+), barely detectable; (+), equivalent to that of matched normal tissue; (++), moderately overexpressed; (+++), highly overexpressed; (++++), very highly overexpressed; relative to that of matched normal tissue. *The level of p16 protein expressed both by the normal and tumor tissues of COBRC13 and COBRC15 was equivalent but barely detectable.

Table 9 Expression of Rb, p16 and cyclin D1 proteins in breast cancer cell lines

Cell line	Rb protein	p16 protein	cyclin D1 protein
MCF-12A	+	-	+
MDA-MB-157	+	+	+
COLO 742	+	+	+++
MDA-MB-415	+	+	+++++

The expression of Rb, p16 and cyclin D1 proteins in normal and tumor breast epithelial cell lines was assessed by immunoblot analysis. The presence or absence of Rb and p16 protein expression was indicated by a (+) or a (-) respectively. The levels of cyclin D1 protein in the tumor cell lines were compared to that in MCF-12A and indicated as follows: (+), equivalent to that of MCF-12A; (+++), approximately 5-fold overexpression; (+++++), approximately 10-fold overexpression.